METHODOLOGY

The study involved 120 patients with invasive ductal carcinoma of the pancreas who underwent radical resection at Keio University Hospital (Tokyo, Japan) during a 20-year period from 1976 to 1995. The clinical records of these patients were retrospectively reviewed to determine the prognostic utility of the preoperative and postoperative platelets counts in regard to survival. Fifteen patients whose preoperative platelets count data were missing were excluded. As a result, data of 105 patients, 75 men and 30 women, were analyzed in this study. Their ages ranged from 29 to 86 years old, and their mean age was 61.2 years old.

Laboratory data were collected on admission and at intervals of approximately 1 month after the operation. Complete blood counts, including platelets counts, were obtained in the hospital laboratory and analyzed.

The final histopathological diagnoses were: tubular adenocarcinoma, well differentiated: 19 (18.1%), tubular adenocarcinoma, moderately differentiated: 63 (60.0%), tubular adenocarcinoma, poorly differentiated: 6 (5.7%), papillary adenocarcinoma: 10 (9.5%), adenosquamous carcinoma: 3 (2.9%), and others: 4 (3.8%). At surgery, pancreatoduodenectomy (PD) or pylorus-preserving pancreatoduodenectomy (PpPD) was basically performed to treat tumors of the head of the pancreas, while distal pancreatectomy plus splenectomy (DP+SP) was performed for tumors of the body and the tail of the pancreas. Dissection of regional lymph nodes was performed in all cases. Patients with a large tumor located in the head and body of the pancreas were treated by total pancreatectomy plus splenectomy (TP+SP) with dissection of regional lymph nodes.

All patients were followed up closely at our hospital after discharge. The follow-up period ranged from a minimum of one month to a maximum of 191 months. Ninety-seven cases could be evaluated 1 month after the operation. Laboratory data were collected, and computed tomography or ultrasonography was performed at intervals of approximately 1 month, 3 months, and 6 months and then every 6 months after discharge.

In accordance with other studies (9,10), we defined non-elevated platelets counts as a count of less than $40x10^4$/mL and thrombocytosis as a count of $40x10^4$/mL or higher. The patients were also classified by changes in platelets counts between the preoperative and postoperative period. In group A, the preoperative and postoperative platelets counts were both $40x10^4$/mL or higher. In group B, the preoperative platelets count was $40x10^4$/mL or higher and the postoperative one was less than $40x10^4$/mL. In group C, the preoperative platelets count was less than $40x10^4$/mL and the postoperative one was $40x10^4$/mL or higher, and in group D the preoperative and postoperative platelets counts were both less than $40x10^4$/mL.

Since splenectomy is often associated with an increase of postoperative platelets counts, to accurately evaluate the influence of postoperative platelets counts on the outcome, it was necessary to analyze the cumulative survival rates of patients who had undergone PD (without SP).

Staging was determined based on histopathological findings according to the UICC pTNM classification of malignant tumors.

Some patients had undergone intraoperative radiotherapy (IOR) and/or postoperative chemotherapy. IOR was performed on 37 patients (35.2%) and chemotherapy was performed on 68 patients (43.8%).

As for the outcome, the survival curves of the each group was prepared by the Kaplan-Meier method and analyzed by the log-rank test. Background factors, such as hematological findings and histopathological findings, of the two groups were compared between the group in which the preoperative platelets count was $40x10^4$/mL or higher and the group in which it was less than $40x10^4$/mL by χ^2 test.

RESULTS

Survival Rate of Two Preoperative Platelets Count Groups

The preoperative platelets count was $40x10^4$/mL or higher in 14 (13.3%) patients aged 29-73 years (mean age: 58.3 years old) consisting of nine men and five women. The preoperative platelets count was less than $40x10^4$/mL in 91 patients (67.6%) consisting of 65 men and 26 women, and their age range was 40-86 years (mean age: 62.2 years old).

The cumulative survival rate was significantly poorer in the group with thrombocytosis (n=14) than in the group with non-elevated platelets counts (n=91, $p=0.043$, **Figure 1**). The one-year and three-year survival rates were 28.6% and 0%, respectively, in the patients with preoperative platelets counts $40x10^4$/mL or higher. In the patients with preoperative platelets counts less than $40x10^4$/mL, the survival rates were 45.1% and 23.1%.

Histological Types, Treatment, and Comprehensive Stages

The primary lesion was localized in the head of the

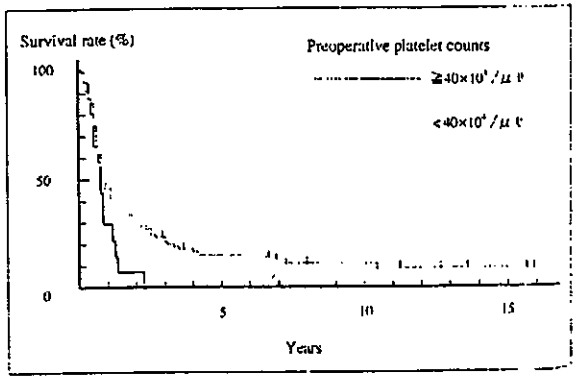

FIGURE 1 Cumulative postoperative survival rates stratified by the preoperative platelets counts of pancreatic cancer patients. The group with thrombocytosis (n=14) had preoperative platelets counts $40x10^4$/mL or higher (——). The group with normal platelets counts (n=91) had counts less than $40x10^4$/mL (——). The outcome of the group with thrombocytosis was significantly poorer than the group with normal platelets counts ($p=0.043$).

pancreas in 88 patients (83.8%) and in the body or tail region in the other 17 (16.2%). PD or PpPD had been performed in 70 patients (66.6%), DP+SP in 10 patients (9.5%) and TP+SP in 25 (23.8%). In the group with preoperative platelets counts of 40x10⁴/mL or higher, PD or PpPD had been performed in nine (64.3%), DP+SP in one (7.1%) and 4 patients (28.6%) had undergone TP+SP. In the group with counts less than 40x10⁴/mL, PD or PpPD had been performed in 61 patients (67.0%), DP+SP in nine (9.9%) and TP+SP in 21 (23.1%). There were no significant differences in the operations.

In the group with preoperative platelets counts of less than 40x10⁴/mL, 29 patients (31.9%) had received intraoperative radiotherapy and 62 patients (68.1%) did not. In the group with 40x10⁴/mL or higher counts, eight patients (57.1%) underwent intraoperative radiotherapy and six patients (42.9%) did not. In the group with preoperative platelets counts of less than 40x10⁴/mL, 49 patients (53.9%) underwent intraoperative and/or postoperative chemotherapy and 42 patients (46.1%) did not. In the group with preoperative platelets counts of 40x10⁴/mL or higher, 10 patients (71.4%) had undergone intraoperative and/or postoperative chemotherapy and four patients (28.6%) had not. There were no significant differences in the ratio of patients who had undergone radiotherapy and/or chemotherapy between the group with thrombocytosis and the group with non-elevated platelets counts (radiotherapy: $p=0.065$, chemotherapy: $p=0.22$).

There were no significant differences in sex ratio, age, histological type, or site of primary lesion between the group with preoperative platelets counts 40x10⁴/mL or higher and the group with less than 40x10⁴/mL, and there were no significant differences in rate of those who had received adjuvant therapy, such as IOR and chemotherapy. The comprehensive stages were compared between the two groups, and no significant differences were observed **(Table 1)**.

Hematological Findings

The preoperative coagulation system, fibrinolysis system, and peripheral blood cells were compared between the patients with thrombocytosis and with non-elevated platelets counts. The PT percentage, aPTT, FDP, hematocrit, hemoglobin, and neutrophil values were not significantly different **(Table 2)**.

Histopathological Findings

Histopathological findings in the resected primary tumors were compared between patients with thrombocytosis and patients with non-elevated platelets count **(Table 3)**. There were no differences between the two groups in primary tumor (T), metastases at regional lymph nodes (N), and distant metastases (M).

Changes in Platelets Counts after Surgery

The patients were classified into four groups (A to D) according to changes in platelets counts. Group A consisted of seven of the 105 patients (6.7%), group B of seven patients (6.7%), group C of 15 patients (14.3%), and group D of 67 patients (63.8%). In group A and group B, the one-year and three-year survival rates were 28.6% and 0%. In group C, the one-year and three-year survival rates were 13.3% and 0%. On the other hand, the one-year and three-year survival rates were 52.2% and 28.4% in group D.

The survival rates were clearly lower in groups A, B and C, compared with group D. There were no significant differences in outcome between group A and

	Preoperative platelets count (x10⁴/mL)			
	All cases	≥40	<40	p
Patients	105	14	91	
Sex				
Men:Women	75:30	9:5	65:26	0.29
Age (yr)	29-86	29-73	40-86	0.21
Mean	61.2	58.3	62.2	
Histological type				
Tubular adenocarcinoma well.	19	1	18	0.46
Tubular adenocarcinoma mod.	63	9	54	
Tubular adenocarcinoma por.	6	1	5	
Papillary adenocarcinoma	10	3	7	
Adenosquamous carcinoma	3	0	3	
Others	4	0	4	
Area				
Pancreatic head	88 (83.8%)	12 (85.7%)	76 (83.5%)	0.84
Pancreatic body	17 (16.2%)	2 (14.3%)	15 (16.5%)	
Operation				
(Pp) PD	70 (66.6%)	9 (64.3%)	61 (67.0%)	0.88
DP+SP	10 (9.5%)	1 (7.1%)	9 (9.9%)	
TP+SP	25 (23.8%)	4 (28.6%)	21 (23.1%)	
IOR				
Done	37 (35.2%)	8 (57.1%)	29 (31.9%)	0.065
Not	68 (64.8%)	6 (42.9%)	62 (68.1%)	
Chemotherapy				
Done	59 (56.2%)	10 (71.4%)	49 (53.9%)	0.22
Not	46 (43.8%)	4 (28.6%)	42 (46.1%)	
Pathological Classification pTNM stage				
I	8 (7.6%)	0	8	0.19
II	14 (13.3%)	1	14	
III	49 (46.7%)	10	38	
IV A	34 (32.4%)	3	31	
IV B	0	0	0	

Background of the study population of patients with invasive ductal carcinoma of the pancreas described by sex, age, Histological type, extent of the tumor, surgical procedure performed, intraoperative radiotherapy (IOR), chemotherapy, stage, and comparison of clinicopathological variables between preoperative platelets count groups.

		Preoperative platelets count (x10⁴/mL)		
		≥40	<40	p
aPPT	sec.	31.45	31.5	0.97
PT %	%	100	80.5	0.14
FDP	ng/dL	102	108.3	0.87
Hct	%	36.4	37.1	0.31
Hb	g/dL	12.3	12.4	0.24
Neutrophil	%	63.5	66.8	0.71

Comparison of laboratory results between preoperative platelets count groups. There were no significant differences in laboratory results without WBC count. The difference may not have been significant clinically because the counts were within the normal range in both groups.

TABLE 3. Histopathological Analysis

		Preoperative platelets count (x10⁴/mL)			
		All cases	≥40	<40	p
T	1	4 (3.8%)	0 (0%)	4 (4.4%)	0.54
Primary tumor	2	10 (9.5%)	1 (7.1%)	9 (9.9%)	
	3	57 (54.3%)	10 (71.4%)	47 (51.6%)	
	4	34 (32.4%)	3 (21.4%)	31 (34.1%)	
N	0	31 (29.5%)	2 (14.3%)	29 (31.9%)	0.18
Regional lymph nodes	1	74 (70.5%)	12 (85.7%)	62 (68.1%)	
M	0	105 (100%)	14 (100%)	91 (100%)	-
Distant metastasis	1	0 (0%)	0 (0%)	0 (0%)	

Histopathological characteristics of the two groups. These findings were evaluated according to the UICC pTNM classification of malignant tumors. There were no significant differences between the two preoperative platelets count groups.

group B, group A and group C, and between group B and group C. However, there was a significant difference in outcome between group B and group D (p=0.045), and between group C and group D (p=0.002) **(Figure 2).**

Survival Rate in the Groups Without Splenectomy

Cumulative survival rates after PD or PpPD were analyzed to accurately evaluate the influence of the postoperative platelets count by eliminating the effects of splenectomy. Seventy patients were underwent PD or PpPD (66.6%). Nine patients had postoperative platelets counts of 40x10⁴/mL or higher and 61 had counts of less than 40x10⁴/mL. The outcome was found to be significantly poorer in the group in which the postoperative platelets count was 40x10⁴/mL or higher compared with the group in which it was less than 40x10⁴/mL (p=0.032, **Figure 3**).

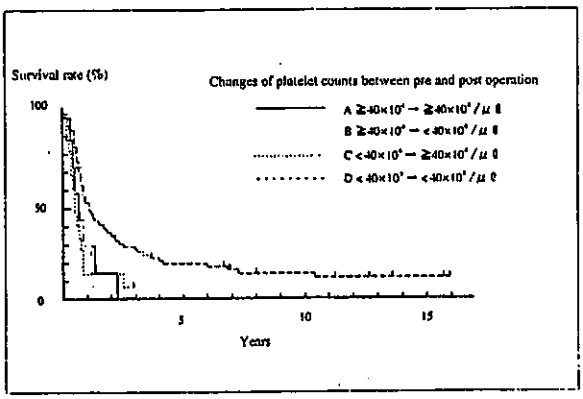

FIGURE 2 Patients were classified according to changes in platelets count, and their outcome was analyzed. Group A had preoperative and postoperative platelets counts 40x10⁴/mL or higher (n=7 ——). Group B had preoperative platelets counts 40x10⁴/mL or higher and postoperative counts less than 40x10⁴/mL (n=7). Group C had preoperative platelets counts less than 40x10⁴/mL and postoperative counts 40x10⁴/mL or higher (n=15 ——). Group D had preoperative and postoperative platelets counts 40x10⁴/mL (n=67 ----). There was a significant difference in outcome between group B and group D (p=0.045), group C and group D (p=0.002). The outcome of group D was obviously good compared with the other groups.

Comparison of Disease-free Intervals

The disease-free intervals analyzed by the Kaplan-Meier method were compared between the group in which the preoperative platelets count was 40x10⁴/mL or higher and the group in which it was less than 40x10⁴/mL. The mean disease-free interval was 4.9 months in the 40x10⁴/mL or higher group and 46.5 months in the less than 40x10⁴/mL group, showing that recurrence was significantly delayed in the less than 40x10⁴/mL group (p=0.005, **Figure 4**).

DISCUSSION

We have shown that thrombocytosis is associated with a poor outcome and earlier tumor recurrence in patients with pancreatic cancer. The outcome of patients with invasive ductal carcinoma of the pancreas was significantly poorer in the group in which the preoperative platelets count was 40x10⁴/mL or higher compared with in the group without thrombocytosis. These findings are consistent with past reports of a poorer outcome in patients with cancer of the lung, uterine cervix, endometrial carcinoma, and renal cancer with increased preoperative platelets counts (7-9,16). The mean disease-free interval in the 40x10⁴/mL count group was also significantly shorter.

In our patients, the overall survival rate was 42.9% after 1 year, 20.0% after 3 years and 12,9% after 5 years, and similar to the mean survival rates of patients with invasive ductal carcinoma of the pancreas treated by radical pancreatectomy in Japan (1 year: 51.2%, 3 years: 25.1%, 5 years: 18.2%) (17). Moreover, the proportions of comprehensive stages, histological types, and types of selected operative procedures were also similar to the means for patients treated in Japan. Thrombocytosis has been found in association with several carcinomas (7,18,19). We found thrombocytosis in 13.3% of our patients with pancreatic cancer. This thrombocytosis rate is similar to the rates found in patients with cancer of other organs, endometrial cancer and cervical cancer (20-23). These above data indicate that our patients were not particular for other reported cases.

Comparison of the clinical background data of the group in which the preoperative platelets count was 40x10⁴/mL or higher and the group in which it was less than 40x10⁴/mL showed no significant differences in age, sex ratio, histological type, or peripheral blood coagulation factors, and there were no differences in any of the pathological diagnostic factors, i.e. T (primary tumor), N (metastases of regional lymph nodes), or M (distant metastases). There were no significant differences in tumor locations or surgical procedure between the preoperative thrombocytosis group and the non-elevated platelets count group, and there were no significant differences in the rate of patients subjected to adjuvant therapy, such as intraoperative radiotherapy or intra- or postoperative chemotherapy. Despite the absence of significant differences in any of the parameters, our investigation showed that the

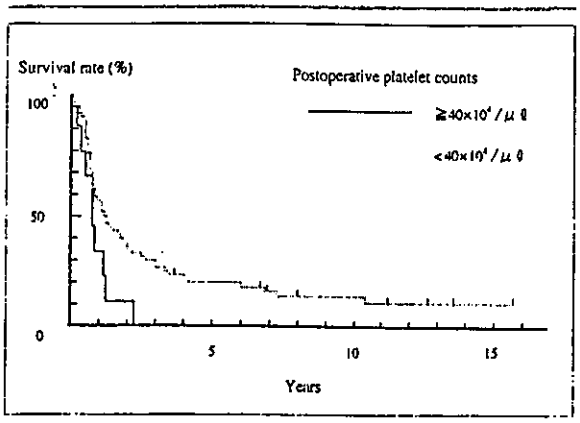

FIGURE 3 Cumulative survival rate after pancreatoduodenectomy (without the effect of splenectomy) stratified by the preoperative platelets counts. The outcome was significantly poorer in the group in which the postoperative platelets count was 40x10⁴/mL or higher (n=9 ——) than in the group in which it was less than 40x10⁴/mL (n=61 ·····) (p=0.032).

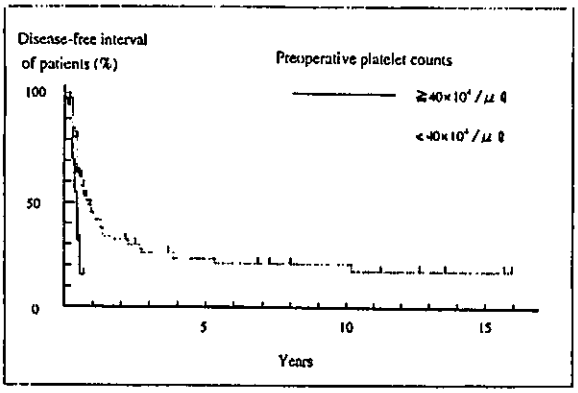

FIGURE 4 Comparison of postoperative disease-free intervals between the two preoperative platelets count groups. Recurrences occurred significantly later in which the preoperative platelets count was less than 40x10⁴/mL group (·) than the group in which the preoperative platelets count was 40x10⁴/mL or higher ( ) (p=0.005).

outcome of patients with invasive ductal carcinoma of the pancreas whose preoperative platelets count was 40x10⁴/mL or higher was significantly poorer than that of without thrombocytosis.

Since several studies have reported on the potential hazard of intraoperative hematogenous dissemination of tumor cells (12-14), and Kobayashi *et al.* reported the risk of shedding cancer cells into a portal vein while manipulating pancreatic head lesions during pancreatoduodenectomy for pancreatic cancers (15), the relation between outcome and changes in platelets counts after surgery may be important. The outcome was significantly poorer in the group in which either preoperative or postoperative counts were 40x10⁴/mL or higher as compared with the group in which preoperative and postoperative platelets counts were both less than 40x10⁴/mL. Splenectomy is often associated with an elevation of postoperative platelets count, and splenectomy was performed in patients with pancreatic cancer located in the body or tail of the pancreas. Thus, to accurately investigate the influence of postoperative platelets counts on the outcome, we analyzed the survival rates of patients who had undergone PD or PpPD (without splenectomy) and compared them with those of the group with high and low postoperative platelets counts. Seventy patients were underwent PD or PpPD (66.6%). The outcome was significantly poorer in the group in which the postoperative platelets count was 40x10⁴/mL or higher than in the group in which the postoperative platelets count was less than 40x10⁴/mL. The postoperative platelets count as well as the preoperative platelets count may influence the outcome of patients with pancreatic cancer.

Schwarz and colleagues reported that splenectomy performed at the time of pancreatectomy in patients with pancreatic cancer led to a poor outcome (24), and they attributed the poorer prognosis after splenectomy to an altered immune response to micrometastatic cancer after removal of splenic immunocompetent cells or different growth characteristics of lymphatic micrometastases in the absence of splenic lymphocytic regulations. We think that splenectomy leads to thrombocytosis in the early postoperative period and may partly lead to a poorer outcome in patients with pancreatic cancer because of an interaction between the increased number of platelets and tumor cells as a result, for example, of expression of extracellular matrix degenerating enzymes by tumor cells.

The pathophysiologic mechanism underlying thrombocytosis in malignant disease is still unclear. The process of megakaryocytopoiesis, which leads to the production of platelets, is complex and regulated by a number of circulating humoral factors. However, maturation of megakaryocytes is regulated primarily by interleukin-6 (IL-6). IL-6 and macrophage colony-stimulating factor (M-CSF) may be responsible for the development of cancer related thrombocytosis (25,26). Moradi *et al.* showed that patients with ovarian cancer have elevated levels of IL-6 in serum and ascitic fluid. (27). In a recent report, Okada *et al.* described that the serum IL-6 level in patients with pancreatic cancer was significantly increased compared to patients with pancreatitis (28).

Interactions between tumor cells and platelets have been attracting attention. Many tumor cells secrete thrombin or a thrombin-activating factor, leading to platelets activation (29), while activated platelets secrete adherent proteins, such as fibrinogen, fibronectin, factor V and VIII, and thrombospondin, and strengthen the adherence between tumor cells and platelets or between tumor cells and endothelial cells. It has been reported that the rate of distant metastases of tumor cells that strongly aggregate platelets is high and that the metastases rate of tumor cells that weakly aggregate platelets is low, suggesting that the platelets aggregation activity of tumor cells correlates with the metastases rate (30). It has recently been reported that the *in vitro* infiltration activity of tumor cells is increased by co-culture with platelets (31). Moreover, the expression level of matrix metalloprotease, MMP-9, by human mammary tumor cells has been reported to be increased by co-culture of cancer cell line with platelets (32). Angiogenesis is a pre-

requisite for tumor growth and metastases. Tumor angiogenesis may be mediated by several angiogenic factors such as vascular endothelial growth factor (VEGF), platelets-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), which are expressed more frequently in some malignant tumors than in normal tissue (33), and are also secreted from activated platelets (34,35). They have been also associated with liver metastases from various primary tumor types (36), and the expressions of VEGF and PDGF have been established as important prognostic indicators for many tumor types (37,38). These factors from platelets as well as from tumor tissues may be associated with the shorter interval until recurrence observed in the group with thrombocytosis.

In order to analyze the mechanism by which platelets counts influence the outcome of patients with pancreatic cancer, further studies in this field such as on interactions between tumor cells and platelets, invasiveness of tumor cells and expression of gelatinase by tumor cells in the presence of platelets, and on the effect of antiplatelets agents on their interaction should also be investigated. Inhibition of platelets function by antiplatelets drugs may improve the outcome of cancer patients.

The cause of the poor outcome of patients with preoperative and postoperative thrombocytosis remains unclear. However, as shown in this study, preoperative and postoperative platelets counts are closely related to the outcome of patients with pancreatic cancer.

REFERENCES

1 **Held-Warmkessel J, Volpe H, Waldman AR:** Treatment for pancreatic cancer. Clin J Oncol Nurs 1998; 2(4):127-134.
2 **Howard TJ:** Pancreatic adenocarcinoma. Curr Probl Cancer 1996; 20(5):281-328.
3 **Honn KV, Onoda JM, Menter DG, Cavanaugh PG, Crissman JD, Taylor JD, Sloane BF:** Platelets-tumor cell interaction as targets for antimetastatic therapy. In: Honn KV, Power WE, Sloane BF (Eds.). Mechanism of Cancer Metastases. Boston: Martinus Nijhoff Publishing, 1986; pp. 117-144.
4 **Gasic GJ, Gasic TB, Galanti N, Johnson T, Murphy, S:** Platelets-tumor-cell interactions in mice. The role of platelets in the spread of malignant disease. Int J Cancer 1973; 11(3):704-718.
5 **Pearlstein E, Ambrogio C, Karpatkin S:** Effect of antiplatelets antibody on the development of pulmonary metastases following injection of CT26 colon adenocarcinoma, Lewis lung carcinoma, and B16 amelanotic melanoma tumor cells into mice. Cancer Res 1984; 44(9):3884-3887.
6 **Honn KV, Cicone B, Skoff A:** Prostacyclin: a potent antimetastatic agent. Science 1981; 212(4500):1270-1272.
7 **Costantini V, Zacharski LR, Moritz TE, Edwards RL:** The platelets count in carcinoma of the lung and colon. Thromb Haemost 1990; 64:501-505.
8 **Rodriguez GC, Clarke-Pearson DL, Soper JT, Berchuck A, Synan I, Dodge RK:** The negative prognostic implications of thrombocytosis in women with stage IB cervical cancer. Obstet Gynecol 1994; 83(3):445-448.
9 **Moller-Pedersen L, Milman N:** Prognostic significance of thrombocytosis in patients with primary lung cancer. Eur Respir J 1996; 9:1826-1830.
10 **Menczer J, Geva D, Schejter E, Zakut H:** Elevated platelets count in patients with endometrial carcinoma: correlation with selected prognostic factors and with survival. Int J Gynecol Cancer 1996; 6:463-554.
11 **Kamisawa T, Isawa T, Koike M, Tsuruta K, Okamoto A:** Hematogenous metastases of pancreatic ductal carcinoma. Pancreas 1995; 11(4):345-349.
12 **Wong IH, Lau WY, Leung T, Yeo W, Johnson PJ:** Hematogenous dissemination of hepatocytes and tumor cells after surgical resection of hepatocellular carcinoma: a quantitative analysis. Clin Cancer Res 1999; 5(12):4021-4027.
13 **Yamashita JI, Kurusu Y, Fujino N, Saisyoji T, Ogawa M:** Detection of circulating tumor cells in patients with non-small cell lung cancer undergoing lobectomy by video-assisted thoracic surgery: a potential hazard for intraoperative hematogenous tumor cell dissemination. J Thorac Cardiovasc Surg 2000; 119(5):899-905.
14 **An XY, Egami H, Hayashi N, Kurusu Y, Yamashita JI, Ogawa M:** Clinical significance of circulating cancer cells in peripheral blood detected by reverse transcriptase-polymerase chain reaction in patients with breast cancer. Tohoku J Exp Med 2001; 193(2):153-162.
15 **Kobayashi S, Asano T, Ochiai T:** A proposal of no-touch isolation technique in pancreatoduodenectomy for periampullary carcinomas. Hepatogastroenterology 2001; 48(38):372-374.
16 **O'Byrne KJ, Dobs N, Propper D, Smith K, Harris AL:** Vascular endothelial growth factor platelets counts, and prognosis in renal cancer. Lancet 1999; 353(9163):1494-1495.
17 **Yamamoto M, Ohashi O, Saitoh Y:** Japan Pancreatic Cancer Registry: current status. Pancreas 1998; 16(3):238-342.
18 **Carrington PA, Carr TF, Stevens RF, Evans DIK:** Thrombocytosis associated with solid tumors in children. Peditatr Hematol Oncol 1992; 9:289-291.
19 **Marchasin S, Wallerstein RO, Aggeler PM:** Variation of the platelets count in disease. Calif Med 1964; 101:95.
20 **Hernandez E, Lavine M, Dunton CJ, Gracely E, Parker J:** Poor prognosis associated with thrombocytosis in patients with cervical cancer. Cancer 1992; 69:2975-2977.
21 **Lopes A, Daras V, Cross PA, Robertson G, Beynon G, Monaghan JM:** Thrombocytosis as a prognostic factor in women with cervical cancer. Cancer 1994; 74:90-92.
22 **Hernandez E, Heller PB, Whitney C, Diana K, Delgado G:** Thrombocytosis in surgically treated stage IB squamous cell cervical carcinoma (a gynecologic Oncology Group study). Genecol Oncol 1994; 55:328-332.
23 **Gucer F, Moser F, Tamussino K, Reich O, Haas J, Arikan G, Petru E, Winter R:** Thrombocytosis as a prognostic factor in endometrial carcinoma. Gynecol Oncol 1998; 70(2):210-214.
24 **Shwarz RE, Harison LE, Conlon KC, Klimstra DS, Brennan MF:** The impact of splenectomy on outcomes after resection of pancreatic adenocarcinoma. J Am Coll Surg 1999; 188(5):516-521.
25 **Imai T, Koike K, Kubo T, Kikuchi T, Amano Y, Takagi M, Okumura N, Nakahata T:** Interleukin-6 supports human megakaryocytic proliferation and differentiation in vitro. Blood 1991; 78(8):1969-1974.
26 **Ramakrishnan S, Xu FJ, Brandt SJ, Niedel JE, Bast RC Jr, Brown EL:** Constitutive production of macrophage colony-stimulating factor by human ovarian and breast cancer cell lines. J Clin Invest 1989; 83(3):921-926.
27 **Moradi MM, Carson LF, Weinberg B, Haney AF, Twiggs LB, Ramakrishnan S:** Serum and ascitic fluid levels of interleukin-1, interleukin-6, and tumor necrosis factor-alpha in patients with ovarian epithelial cancer. Cancer 1993; 72(8):2433-2440.
28 **Okada S, Okusaka T, Ishii H, Kyogoku A, Yoshimori M, Kajimura N, Yamaguchi K, Kakizoe T:** Elevated serum interleukin-6 levels in patients with pancreatic cancer. Jpn J Clin Oncol 1998; 28(1):12-15.
29 **Ordinas A, Diaz-Ricart M, Almirall L, Bastida E:** The role of platelets in cancer metastases. Blood Coagul Fibrinolysis 1990; 1(6):707-711.

30 **Mahalingam M, Ugen KE, Kao KJ, Klein PA:** Functional role of platelets in experimental metastases studied with cloned murine fibrosarcoma cell variants. Cancer Res 1988; 48(6):1460-1464.
31 **Belloc C, Lu H, Soria C, Fridman R, Legrand Y, Menashi S:** The effect of platelets on invasiveness and protease production of human mammary tumor cells. Int J Cancer 1995; 60:413-417.
32 **Lindenmeyer F, Legrand Y, Menashi S:** Upregulation of MMP-9 expression in MDA-MB231 tumor cells by platelets granular membrane. FEBS Lett 1997; 418:19-22.
33 **Anan K, Morisaki T, Katano M, Ikubo A, Kitsuki H, Uchiyama A, Kuroki S, Tanaka M, Torisu M:** Vascular endothelial growth factor and platelets-derived growth factor are potential angiogenic and metastatic factors in human breast cancer. Surgery 1996; 119(3):333-339.
34 **Mohle R, Green D, Moore MA, Nachman RL, Rafii S:** Constitutive production and thrombin-induced release of vascular endothelial growth factor by human megakaryocytes and platelets. Proc Natl Acad Sci USA 1997; 94(2):663-668.
35 **Banks RE, Forbes MA, Kinsey SE, Stanley A, Ingham E, Walters C, Selby PJ:** Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements and cancer biology. Br J Cancer 1998; 77(6):956-964.
36 **Takeda A, Stoeltzing O, Ahmad SA, Reinmuth N, Liu W, Parikh A, Fan F, Akagi M, Ellis LM:** Role of angiogenesis in the development and growth of liver metastases. Ann Surg Oncol 2002; 9(7):610-616.
37 **Knoll MR, Rudnitzki D, Sturm J, Manegold BC, Post S, Jaeger TM:** Correlation of postoperative survival and angiogenic growth factors in pancreatic carcinoma. Hepatogastroenterology 2001; 48(40):1162-1165.
38 **Jinno K, Tanimizu M, Hyodo I, Nishikawa Y, Hosokawa Y, Doi T, Endo H, Yamashita T, Okada Y:** Circulating vascular endothelial growth factor (VEGF) is a possible tumor marker for metastases in human hepatocellular carcinoma. J Gastroenterol 1998; 33(3):376-382.

FOOTNOTE

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Antitumor effect induced by dendritic cell (DC)-based immunotherapy against peritoneal dissemination of the hamster pancreatic cancer

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Abstract

Establishing a method to control peritoneal dissemination is one of the most pressing issues in the postsurgical treatment of pancreatic cancer. In the present study, we investigated the effect of dendritic cell (DC)-based immunotherapy on peritoneal disseminations of hamster pancreatic cancer cells, PGHAM-1. After the orthotopically inoculation of 2×10^6 PGHAM-1 cells, DC pulsed with PGHAM-1-derived tumor lysates, DC alone or PBS as a vehicle was injected intraperitoneally (i.p.) three times at weekly intervals. The group treated with DC or DC+lysate was found to have smaller disseminated tumors than the vehicle-treated. In addition, mean survival time in the DC+lysate groups was significantly longer than the PBS group. These findings suggested that DC-based immunotherapy might be efficient for the treatment of peritoneal disseminations of the pancreatic cancer.

Keywords: Hamster DC; Pancreatic cancer; Orthotopic model; Peritoneal dissemination; DC migration; Cytotoxic T-lymphocyte

1. Introduction

In almost all cases of pancreatic cancer patients, because of liver metastasis, peritoneal dissemination and local recurrence in the early postoperative stage, even macroscopically radical operations have not been successful in improving the prognosis of

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pancreatic cancer. Peritoneal dissemination is the most important factor in the prognosis of pancreatic cancer patients. Therefore, a therapeutical approach to control peritoneal dissemination is urgently required in clinical oncology.

Recently, using several DC-based immunotherapeutic approaches against intractable cancers, some positive results were reported for melanoma [1], kidney [2] and prostate cancers [3], lymphoma [4] and colorectal cancers [5]. However, few clinical approaches to the treatment of peritoneal dissemination have been successful, because control of it is very difficult in the devastating conditions of terminal cancer and the poor self-immune response of such patients. Previously, our group already demonstrated that hamster bone marrow (BM)-derived DCs loaded with tumor lysate could induce tumor-specific cytotoxic T lymphocyte (CTL) activity and a significant anti-tumor response in subcutaneously pancreatic cancer-bearing hamsters [6].

In the present study, based on the promising therapeutical effect on a subcutaneous tumor, we have focused on peritoneal dissemination, the worst of the local progressions or recurrences after surgery in pancreatic cancer resulting in death, and investigated the therapeutical effect of DC treatment on pancreatic cancer-induced peritoneal disseminations in hamsters in terms of tumor weight and survival time.

2. Materials and methods

2.1. Hamsters and cell lines

Specific pathogen-free 3 to 4-week-old female Syrian hamsters were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed under pathogen-free conditions. All animal used in this study were cared for and used humanely according to the principles of laboratory animal care (NIH publication No. 85-23, revised 1985) and the Guidelines for animal experiments of the National Cancer Center, Tokyo. The PGHAM-1 hamster pancreatic cancer cell line was kindly provided by Dr Uchida (Nippon Medical School, Tokyo, Japan), and maintained in Dulbecco's modified Eagle's medium (DMEM) (SIGMA, St. Louis, MO) containing 10% fetal bovine serum (Invitrogen, Rockville, MD), 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. The DDT1MF-2 cell line, Syrian hamster leiomyosarcoma cells and the YAC-1 cell line were purchased from the American Type Culture Collection (Manassas, VA).

2.2. Culture of bone marrow-derived DCs and antigen pulsing

The method of BM-derived DC culture and pulsing with tumor lysates was previously described [6]. Briefly, unfractioned BM cells were cultured with mGM-CSF and mIL-4 at a concentration of 2×10^6 cells per ml in a 24-well culture plate for 7 days. Non-adherent, DC-enriched cell populations were collected by gentle pipetting. Then 1×10^7 DC-enriched cells resuspended in Opti-MEM medium and 2 mg of tumor lysate from PGHAM-1 cells mixed with 50 μg of *N*-[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP) were incubated 4 h at 37 °C in a water bath with constant gentle shaking. The DCs were washed three times with PBS (−) and used for intraperitoneal injection.

2.3. Tracking of GFP (green fluorescence protein)-labeled BM-derived DCs in vivo

The method of construction of the recombinant adenoviral vector containing the GFP gene and generation of adenovirus was described before [7]. After a 7-day-culture, DCs were harvested and resuspended at 5×10^6 cells per ml. Supernatant of adenovirus containing GFP cDNA (AdGFP) or mock virus was added to DCs at multiplicity of infection (MOI) of 100 and incubated for 2 h. After two washes, the cells were incubated for another 24 h with GM-CSF and IL-4. 1×10^7 Hamster DCs transduced with GFP cDNA (DC-GFP) or mock DCs were resuspended in 200 ul of PBS (−) and injected intraperitoneally (i.p.) into the hamsters. After 24 h, omentum, mesenteric and paraaortic lymphnodes were resected and single cell suspensions were made. Fluorescence-positive cells were measured using a FACSCalibur (Beckton-Dickinson, SanDiego, CA).

2.4. Adenoviral-retroviral sequential infection

PGHAM1 cells with 50% confluency on a 6 cm culture plate were infected with the adenovirus containing a human amphotropic retrovirus receptor cDNA (1×10^8 PFU) for an hour [7]. After a change of medium to DMEM supplemented with 10% FBS, they were incubated at 37 °C overnight. Then they were infected with the retrovirus containing cDNAs of EGFP (Clontech) and Ampho [8] by adding viral stock with polybrene (8 μg/ml) (adenoviral-retroviral sequential infection method; manuscript in preparation). At 2 h after the infection, the medium was replaced with DMEM supplemented with 10% FBS and incubated overnight. Blasticidin (108 μg/ml) was added to the medium and maintained until colonies were formed. GFP-expressing PGHAM-1 cells were used for the DC-based treatment model in animal experiments.

2.5. Animal experiments

The cohorts consisted of hamsters injected with PBS (−), DCs or DCs+lysate. On day 0, all hamsters were inoculated into the pancreas (orthotopically) with 2×10^6 non-transduced or GFP gene-transduced PGHAM-1 cells. On day 3, 9 hamsters per group were injected i.p. with PBS (−), 1×10^7 DCs, or 1×10^7 tumor lysate-pulsed DCs per an animal. The injections of vehicle or DCs were repeated 2 more times at weekly intervals. On day 24, one week after the final injection, three hamsters from each group were sacrificed for the measurement of resected tumor weight and metastatic nodule numbers and the investigation of CTL activity using spleen cells. A small amount of resected tumor from each group was also used for histological examinations. All 6 remaining hamsters were monitored until death, and the therapeutical effect on survival time was analyzed. The therapeutical effect on GFP-expressing tumor-inoculated hamsters was investigated using a digital CCD camera (Hamamatsu Photonics K.K., Hamamatsu, Japan).

2.6. CTL assays

Spleens were harvested from three hamsters per group on day 24, 7 days after the last DC injection, and used for the assay of CTL activity. The method of CTL assay was described before [6]. Briefly, spleen cells (5×10^6/ml) were co-cultured with irradiated (18,000 rads) PGHAM-1 cells (5×10^5/ml) in 6-well plates for 5 days. Restimulated spleen cells were used as effectors for the ^{51}Cr release assay. PGHAM-1 cells, DDT1MF-2 cells as an irrelevant tumor or YAC-1 cells as a target of NK cells were labeled with $Na_2\,^{51}CrO_4$. The labeled cells were mixed with effector cells at various *E/T* ratios in 96-well flat bottomed culture plates for 4 h. The method of measuring the radioactivity in supernatant was described previously [6].

2.7. Statistical analysis

Statistical differences were analyzed using the two-tailed Student's *t* test. Survival in vivo was analyzed by comparing differences in mean survival times using the Kaplan–Meier method. A comparative analysis of survival times between groups was then made using the Logrank test. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Tracking of GFP-labeled BM-derived DCs in vivo

Almost all (more than 85%) GFP cDNA-transduced hamster DCs were GFP-positive and the MFI was very high, 5147 (data not shown). In vivo, the frequency of fluorescence-positive cells in the lymph node cell suspension was highest in omental lymph node tissue (6.8%, Fig. 1). No significant number of positive cells was seen in other lymph node tissues.

3.2. Inhibition of tumor growth and peritoneal dissemination

On day 24, 7 days after the last DC injection, 3 hamsters from each group were harvested and the remaining tumors were investigated. Tumor weight and the number of metastatic nodules were significantly reduced in the groups treated with DC or DC+lysates compared with the PBS group (Table 1). The amount of bloody ascites was also decreased in the groups injected with DC or DC+lysates. In terms of external appearance of hamsters, there was

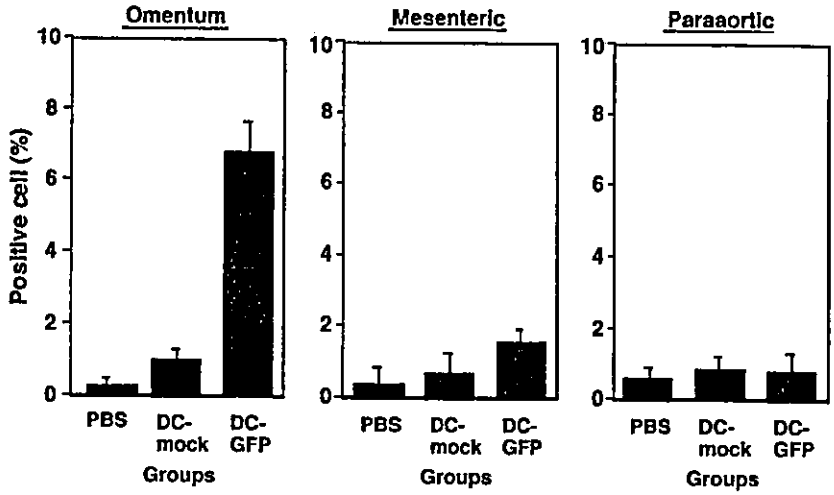

Fig. 1. Tracking of green fluorescence protein (GFP)-labeled BM-derived hamster DCs in vivo. Hamster DCs (1×10^7) transduced with GFP cDNA (DC-GFP) or mock gene (DC-mock) were injected intraperitoneally into hamsters. After 24 h, omentum, mesenteric and paraaortic lymph nodes were harvested and fluorescence-positive cells were measured using a FACSCalibur. Each column represents the mean±SD of lymph node samples from three hamsters.

a marked difference between the PBS group and the treated groups; remarkable abdominal distention was noted in the PBS group, whereas in the treated groups, the abdomen looked flat (data not shown). There was also a significant difference in the number of metastatic nodules and ascites between the DC group and DC+lysate group (Table 1). Additionally, in the GFP-expressing PGHAM-1 tumor treatment model, DC+lysate group showed no significant GFP spot compared with the PBS group, which showed multiple GFP spots in accordance with metastatic lesions (Fig. 2).

3.3. Cytotoxic T cell activity against PGHAM-1 tumors after intraperitoneal injection of BM-derived DCs

The patterns of CTL activity induced by i.p. injected BM-derived DCs and DC pulsed with tumor lysate were consistent with the anti-tumor activity of these groups shown in PGHAM-1 tumor-bearing hamsters. CTL assays revealed that the DC and DC+lysate groups showed more potent CTL activity against PGHAM-1 tumor cells than the PBS control group ($P<0.01$, Fig. 3) at *E/T* ratios greater than 11. In addition, the DC+lysate group exhibited greater CTL activity than the DC group at *E/T* ratios greater than 33 ($P<0.05$). Antibody-based blocking test for CTL activity also demonstrated that anti-CD8 MoAb inhibited moderately but significantly DC-stimulated CTL activity by 32 and 38% in DC alone and DC+lysate group, respectively (data not shown). No significant differences in NK activity were detected. Most importantly, CTL activity against DDT1MF-2 sarcoma cells, another irrelevant tumor syngeneic to Syrian hamsters, was not induced at any treated group. These results suggested that tumor-specific CTL activity may be an important key factor to

Table 1
Inhibition of pancreatic cancer growth and peritoneal dissemination by DC injections

Groups	Tumor weight (g)	No. of tumor nodules	Ascites (ml)
PBS	2.22±0.75	29.0±9.27	16.0±4.48
DC	1.01±0.85 ($P=0.0482$)	10.6±4.72 ($P=0.0058$)	4.55±3.10 ($P=0.0103$)
DC+lysate	0.24±0.39 ($P=0.0061$)	1.60±2.61 ($P=0.0014$)	0 ($P=0.0014$)

The data show the mean±SD from three hamsters. Values in parentheses indicate *P* values for the DC or DC+lysate group compared with the PBS group.

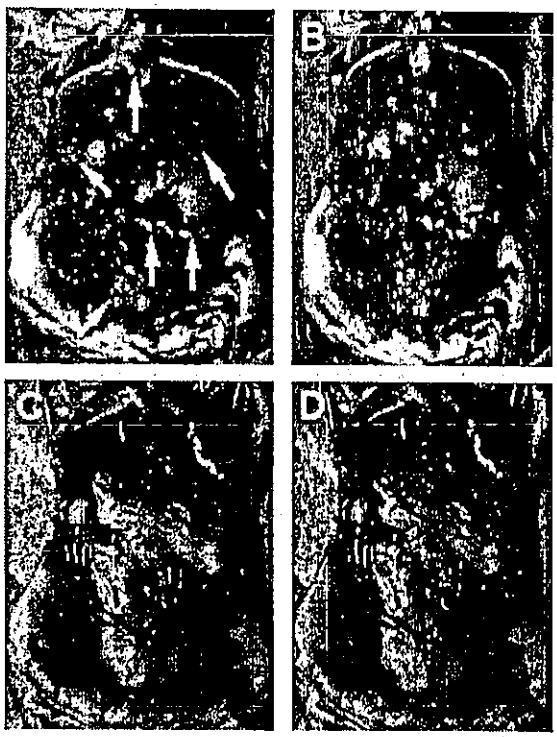

Fig. 2. Remarkable growth suppression of orthotopically inoculated PGHAM-1 tumor after repeated DC vaccinations. (A) and (B) PGHAM-1 cell-inoculated hamster treated with PBS. Multiple tumor nodule disseminations were seen in the peritoneal cavity (arrows). (C) and (D) GFP-positive PGHAM-1 cell-inoculated hamsters treated with DCs+tumor lysates. (A) and (C) regular image, (B) and (D) GFP image overlayed.

the anti-tumor effects of tumor lysate-pulsed DC against PGHAM-1 tumors.

3.4. Histological observations in PGHAM-1 tumors from hamsters treated with tumor lysate-pulsed DCs

PGHAM-1 tumors from hamsters injected with tumor lysate-pulsed DCs showed a remarkable less of viable tumor cells and massive necrosis with heavy infiltration of mononuclear and polymorphonuclear leukocytes (data not shown).

3.5. Survival analysis

All hamsters in the PBS group died within 4 weeks of the inoculation of PGHAM-1 cells. In contrast, almost all hamsters from the treated groups were still alive after 4 weeks (Fig. 4). The mean survival time was significantly longer for the DC and DC+lysate groups than PBS group (DC+lysate 48.8 days, DC 35.0 days versus PBS 25.0 days; $P<0.01$ by Logrank test). In addition, survival in the DC+lysate group was demonstrated to be significantly prolonged compared with the DC group ($P<0.05$).

4. Discussion

To date, several therapeutic attempts to control peritoneal disseminations of pancreatic cancers have

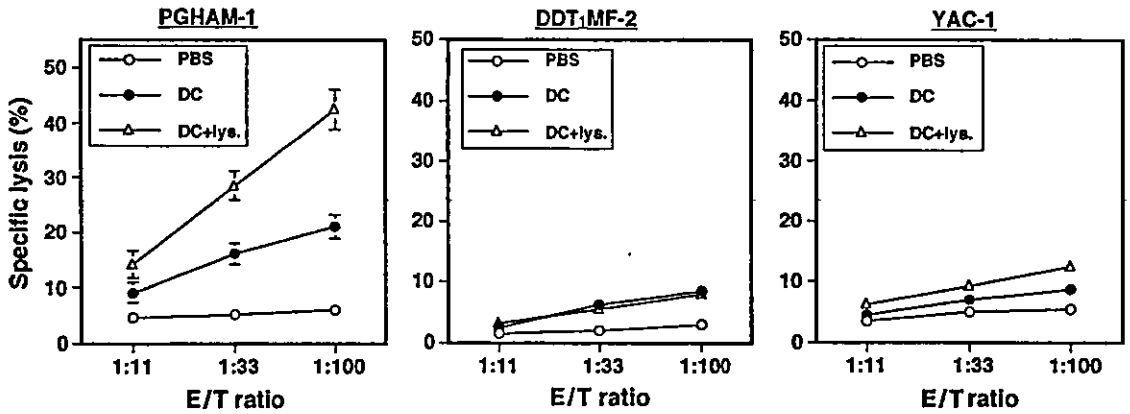

Fig. 3. CTL activity induced by tumor lysate-pulsed DCs in hamsters orthotopically inoculated with PGHAM-1 cells. On day 24, spleen cells were harvested from 3 hamsters per group and pooled. The spleen cell suspension (2.5×10^6 per ml) was co-cultured with 2.5×10^5 per ml of irradiated (180 Gy) PGHAM-1 cells. Restimulated spleen cells were used as effectors in a ^{51}Cr release assay. Killing activities of effector cells at various *E/T* ratios against (A) PGHAM-1 cells, (B) DDT1MF-2 cells and (C) YAC-1 cells are shown. Data show the mean±SD of triplicate samples.

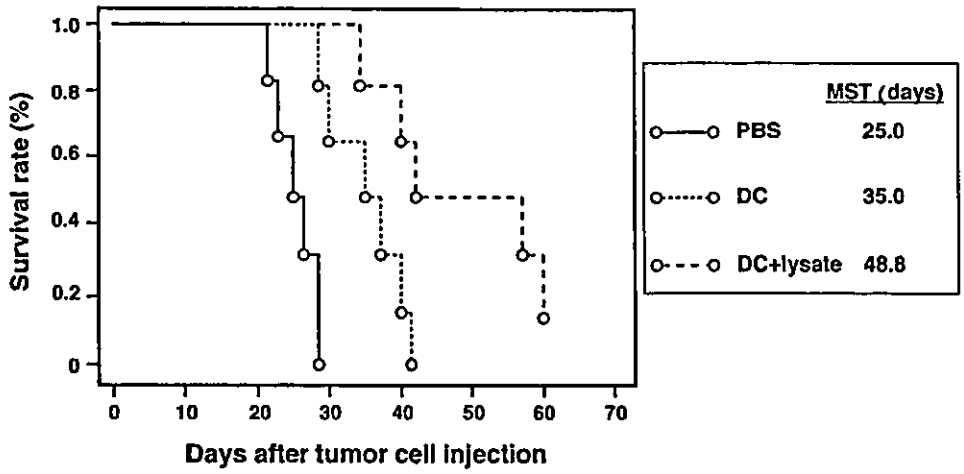

Fig. 4. Prolonged survival of tumor-bearing hamsters treated with DCs pulsed with tumor lysate. All hamsters in the PBS group died within 4 weeks of the inoculation of PGHAM-1 cells. Meanwhile, almost all hamsters from the treated groups survived. Mean survival time was significantly longer for the DC and DC+lysate groups than PBS group (DC+lysate 48.8 days, DC 35.0 days versus PBS 25.0 days; $P<0.01$ by Logrank test).

been reported [9–14]. One group of therapeutic modalities is based on gene therapy. Aoki et al. [9] demonstrated that the herpes simplex virus thymidine kinase (HSV-TK) gene could be transduced intraperitoneally into tumor cells by way of an injection of DNA-lipopolyamine complex, but the feasibility of the gene-delivery system is not clear. Another group uses tumor-antagonizing agents like NK4, a four-kringle fragment of hepatocyte growth factor (HGF). Tomioka et al. [13] reported that NK4 inhibited tumor growth, peritoneal dissemination and ascites accumulation in orthotopic nude mice inoculated with human pancreatic cancer cells into the pancreas, and that the simultaneous targeting at both tumor angiogenesis and the HGF-mediated metastasis might contribute to the anti-tumor effect.

However, few studies of immune cell-based therapeutic approaches based on intensification of the host-defense against pancreatic cancer have been reported. Previously, we demonstrated that hamster BM-derived DCs treated with tumor lysates exhibited obvious anti-tumor effects and caused a tumor-specific CTL induction in hamsters inoculated subcutaneously with syngeneic pancreatic cancer cells [6].

In the present study, as a next step, anti-tumor effect of lysate-treated DCs against peritoneal dissemination of hamster pancreatic cancer was investigated in vivo. To sum up the results of this study, in the group treated with DC+tumor lysates, the growth of orthotopically inoculated tumor cells, PGHAM-1 cells, and ascites production were significantly inhibited. In addition, even in the survival analysis, the injections of DC+lysates had a greater beneficial effect on the survival of tumor-bearing hamsters than PBS or DC injections alone. For another interesting thing, there was an obvious difference of the strength of cellular immune response including CTL or NK activity against tumor cells between subcutaneously (previously described) and the present intraperitoneally tumor-bearing hamsters. Specifically, CTL killing activity against pancreatic cancer cells at the E/T ratio of 100, was much higher in subcutaneous model (more than 60%) than intraperitoneal model (around 40%, Fig. 3). The one of reasons may be the site of tumor or DC vaccination, which is an immunologically important issue. Subcutaneous injection of DCs is an easy way to migrate to regional lymph nodes, trigger the activation of naive T cell, and expand tumor-specific CTLs. On the other hand, intraperitoneal way of DC injection might has some problems that tumor cells in the site or peritoneal macrophages could hinder inducing potent anti-tumor CTLs by means of Th2 cytokines or cell to cell conatct. Second, the difference of target pancreatic cancer cells may be another reason. PGHAM-1 cell

is rapid-growing pancreatic cancer cells with high malignancy compared with HPD1NR cell used in subcutaneous model. Probably, it is easily considered that the induction of positive cellular immune response in the abdominal cavity of PGHAM-1-inoculated hamsters is a difficult protocol without a strong adjuvant effect like DCs.

It can be speculated that the anti-tumor effects on peritoneal dissemination by PGHAM-1 cells were mediated by well-known biological mechanisms; cell migration and Th1 cytokine production. In the first instance, injected DCs transduced with the GFP gene were demonstrated to migrate to the omentum-associated lymphoid tissues, which are anatomically equivalent to milky spots. It is generally considered that omental milky spots act as the first line of host defense in the peritoneal cavity because macrophages, the major cellular component of the spots are dedicated to facilitating phagocytosis and the processing of circulation antigens and foreign bodies generated from the peritoneal cavity [15–17]. In addition, the administration of a biological response modifier (BRM) is reported to activate macrophages in the milky spots and promote their migration into the peritoneal cavity [18]. On the other hand, given that intraperitoneally injected tumor cells tend to invade the milky spots and form tumor nodules, omental milky spots are also closely involved in the dissemination of cancer cells [19]. Taken these findings into consideration, the migration of injected DCs treated with tumor lysates to the milky spots seems to be an immunologically important event which probably triggers the stimulation and expansion of tumor-specific CTL closely linked to a significant anti-tumor effect in vivo.

In the second instance, the PCR analysis revealed that hamster DCs stimulated with tumor lysates upregulated IL-12 gene transcription, that is to say, IL-12 protein production in our study (data not shown). IL-12, a Th1 cytokine generated by antigen-presenting cells like DCs, is demonstrated to have various effects on effector cells and tumor cells; tumor-specific CTL production or NK cell activation is easily promoted and additionally anti-angiogenic effects and FAS upregulation on tumor cells. Also our previous report demonstrating that intratumoral injection of genetically modified mouse DCs producing IL-12 p70 showed a remarkable anti-tumor effect on B16-bearing mice supports the efficacy of IL-12 [20]. In the present study, there might be other mechanisms besides DC migration and IL-12 production mediating the final tumor-specific CTL production and the inhibition of tumor growth.

Even though tumor lysate-pulsed DC therapy is not a novel modality, from a clinical point of view, it is worth applying DC-based immunotherapy to the treatment of intractable pancreatic cancers, especially peritoneal disseminations. It is recommended that this therapeutical approach should be tested in a phase I clinical trial because there is still no effective treatment for advanced pancreatic cancers.

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References

[1] F.O. Nestle, S. Alijagic, M. Gilliet, S. Sun, R. Grabbe, R. Dummer, Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells, Nat. Med. 4 (1998) 328–332.
[2] A. Kugler, G. Stuhler, P. Walden, G. Zoller, A. Zobywalski, P. Brossart, et al., Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids, Nat. Med. 6 (2000) 332–336.
[3] G.P. Murphy, B.A. Tjoa, S.J. Simmons, J. Jarisch, V.A. Bowes, H. Ragde, et al., Infusion of dendritic cells pulsed with HLA-A2-specific prostate-specific membrane antigen peptides: a phase II prostate cancer vaccine trial involving patients with hormone-refractory metastatic disease, The Prostate 38 (1999) 73–78.
[4] F. Hsu, C. Benike, F. Fagnoni, T.M. Liles, D. Czerwinski, B. Taidi, et al., Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells, Nat. Med. 2 (1996) 52–58.
[5] M.A. Morse, Y. Deng, D. Coleman, S. Hull, E. Kitrell-Fisher, S. Nair, et al., A phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed,

autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen, Clin. Cancer Res. 5 (1999) 1331–1338.
[6] Y. Akiyama, K. Maruyama, N. Nara, T. Hojo, J.Y. Cheng, T. Mori, et al., Antitumor effects induced by dendritic cell (DC)-based immunotherapy against established pancreatic cancer in hamsters, Cancer Lett. 184 (2002) 37–47.
[7] K. Maruyama, Y. Akiyama, N. Nara-Ashizawa, T. Hojo, J.Y. Cheng, H. Mizuguchi, Adenovirus-mediated MUC1 gene transduction into human blood-derived dendritic cells, J. Immunother. 24 (2001) 345–353.
[8] R.K. Naviaux, E. Constanzi, M. Haas, I.N. Verma, The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses, J. Virol. 70 (1996) 5701–5705.
[9] K. Aoki, T. Yoshida, N. Matsumoto, H. Ide, K. Hosokawa, T. Sugimura, M. Terada, Gene therapy for peritoneal dissemination of pancreatic cancer by liposome-mediated transfer of herpes simplex virus thymidine kinase gene, Hum. Gene Ther. 8 (1997) 1105–1113.
[10] K. Aoki, S. Furuhata, K. Hatanaka, K.M. Maeda, J.S. Remy, J.P. Behr, et al., Polyethylenimine-mediated gene transfer into pancreatic tumor dissemination in the murine peritoneal cavity, Gene Ther. 8 (2001) 508–514.
[11] H. Kasuya, Y. Nishiyama, S. Nomoto, J. Hosono, S. Takeda, A. Nakao, Intraperitoneal delivery of hrR3 and ganciclovir prolongs survival in mice with disseminated pancreatic cancer, J. Surg. Oncol. 72 (1999) 136–141.
[12] N.J. Lygidakis, N. Spentzouris, M. Theodoracopoulos, G. Dedemadi, K. Gemos, A. Kyriakou, Pancreatic resection for pancreatic carcinoma combined with neo- and adjuvant locoregional targerting immuno-chemotherapy—a prospective randomized study, Hepatogastroenterol. 45 (1998) 396–403.
[13] D. Tomioka, N. Maehara, K. Kuba, K. Mizumoto, M. Tanaka, K. Matsumoto, T. Nakamura, Inhibition of growth, invasion, and metastasis of human pancreatic carcinoma cells by NK4 in an orthotopic mouse model, Cancer Res. 61 (2001) 7518–7524.
[14] Y. Saga, H. Mizukami, M. Suzuki, M. Urabe, A. Kume, T. Nakamura, et al., Expression of HGF/NK4 in ovarian cancer cells suppresses intraperitoneal dissemination and extends host survival, Gene Ther. 8 (2001) 1450–1455.
[15] M. Shimotsuma, T. Takahashi, M. Kawata, K. Dux, Cellular subsets of the milky spots in the human greater omentum, Cell Tissue Res. 264 (1991) 599–601.
[16] M. Shimotsuma, J.W. Shields, M.W. Simpson-Morgan, A. Sakuyama, M. Shirasu, A. Hagiwara, T. Takahashi, Morpho-physiological function and role of omental milky spots as omentum-associated lymphoid tissue (OALT) in the peritoneal cavity, Lymphol. 26 (1993) 90–101.
[17] H.J. Koenen, M.J. Smit, M.M. Simmelink, B. Schuurman, R.H. Beelen, S. Meijer, Effect of intraperotoneal administration of granulocyte/macrophage-colony-stimulating factor in rats on omental milky-spot composition and tumoricidal activity in vivo and in vitro, Cancer Immunol. Immunother. 42 (1996) 310–316.
[18] M. Shimotsuma, M.W. Simpson-Morgan, T. Takahashi, A. Hagiwara, Activation of omental milky spots and milky spot macrophages by intraperotoneal administration of a streptococcal preparation, OK432, Cancer Res. 52 (1992) 5400–5402.
[19] H. Tsujimoto, T. Takahashi, A. Hagiwara, M. Shimomatsu, C. Sakakura, K. Osaki, et al., Site-specific implantation in the milky spots of malignant cells in peritoneal disseminstion: immunohistochemical observation in mice inoculated intraperitonaelly with bromodeoxyuridine-labelled cells, Br. J. Cancer 71 (1995) 468–472.
[20] Y. Akiyama, M. Watanabe, K. Maruyama, F.W. Ruscetti, R.H. Wiltrout, K. Yamaguchi, Enhancement of antitumor immunity against B16 melanoma tumor using genetically modified dendritic cells to produce cytokines, Gene Ther. 7 (2000) 2113–2121.

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Identification of an epigenetically silenced gene, RFX1, in human glioma cells using restriction landmark genomic scanning

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To identify the CpG islands differentially methylated in human glioma, we performed restriction landmark genomic scanning with a CpG methylation-sensitive enzyme. We found 12 spots, the intensity of which was entirely lost or decreased in both the human glioma tissues examined as compared with that in matched normal lymphocytes, indicating aberrant methylation of these CpG islands in gliomas. The expression of RFX1, one of the genes associated with the methylated CpG islands, was frequently decreased in human glioma cell lines and tissues. We also demonstrated that the isolated CpG island located in the seventh intron of the RFX1 gene had enhancer activity and was hypermethylated in all of the glioma tissues and cell lines analysed, but not in normal brains or lymphocytes. Treatment of glioma cells with a demethylating agent, 5-azacytidine, resulted in the expression of RFX1, indicating that the silencing of the RFX1 gene may be attributable to its methylation. RFX1 has been implicated in transcriptional downregulation of the proto-oncogene c-*myc*. By expression of the RFX1 gene, the cellular proliferative activity of glioma cells was suppressed. Taken together, these results suggest that the RFX1 gene may be epigenetically silenced in human gliomas and involved in glioma tumorigenesis.
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Keywords: glioma; RLGS; RFX1; methylation; epigenetics

Introduction

Methylation patterns are established in a tissue-specific manner early during mammalian development, mediated by a combination of demethylation and *de novo* methylation of cytosine residues, primarily at CpG dinucleotides (Melki *et al.*, 1999). The methylation pattern is maintained through subsequent cell divisions by the action of a DNA methyltransferase enzyme. Although most CpG dinucleotides are methylated in normal cells, the CpG islands in gene promoters remain essentially unmethylated. In cancer cells, aberrant hypermethylation occurs in the CpG-rich promoter regions, associating with inactivation of tumor suppressor genes (Melki *et al.*, 1999).

Glioma is the most frequent and malignant human brain tumor. Genetic events in glioma have been extensively analysed, such as EGFR amplification/overexpression, PTEN mutation, p53 mutation, homozygous $p16^{INK4a}$ deletion, and loss of heterozygosity on chromosomes 1p,10q, and 19q (Humphrey *et al.*, 1988; Fults *et al.*, 1992; Sidransky *et al.*, 1992; Di Cristofano *et al.*, 1998; Whang *et al.*, 1998; Rasheed *et al.*, 1999; Yamada and Araki, 2001). Recent studies have suggested that gliomas display a high frequency of methylation, including promoter methylation of the RB1 gene (Hatada *et al.*, 1991; Nakamura *et al.*, 2001), but only a few targets of methylation have been identified so far.

Restriction landmark genomic scanning (RLGS) was developed as a high-speed screening method for identifying multiple genomic alterations (Hatada *et al.*, 1991; Costello *et al.*, 2000; Itano *et al.*, 2000). In RLGS, genomic DNA is digested with specific restriction enzymes and then subjected to direct isotopical end-labeling at the restriction sites. These end-labeled restriction fragments are separated by two-dimensional electrophoresis and detected as discrete spots on an RLGS profile. When a CpG methylation-sensitive enzyme, *Not*I, is used as the landmark enzyme, approximately 2400 DNA fragments representing CpG islands can be displayed by this technique (Smiraglia *et al.*, 1999).

In the present study, we used RLGS with the *Not*I enzyme to detect CpG islands aberrantly methylated in gliomas, and analysed the expression of genes associated with the isolated CpG islands. The expression of RFX1, one of the genes associated with the methylated CpG islands, was decreased in human gliomas. RFX1 is a transcriptional downregulator of the proto-oncogene c-*myc* (Chen *et al.*, 2000). We further analysed the

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relationship between the methylation status and expression of the transcriptional regulator gene RFX1 in gliomas, and the function of this gene as a tumor suppressor.

Results

Identification of methylated CpGs in gliomas

To identify the CpG islands differentially methylated in human glioma, we performed RLGS with a CpG methylation-sensitive enzyme, *Not*I, on two human glioma samples and matched normal lymphocytes. Each RLGS profile had about 2400 spots representing the corresponding loci of genomic DNA (data not shown). In this study, approximately 300 spots were analysed in the central part of the RLGS profiles, where the spots showed the best resolution. We found 12 spots, the intensity of which was entirely lost or decreased in both the human glioma samples examined as compared with that in matched normal lymphocytes (Figure 1). To analyse the sequences of the 12 spots, *Not*I/*Pst*I fragments were isolated from the RLGS gels. CpG islands have a GC content of greater than 50% and the CpG ratio as calculated by the formula, {(number of CpGs)/(number of guanines)(number of cytosines)} (number of nucleotides analysed), is 0.6 or greater (Gardiner-Garden and Frommer, 1987). In this study, all of the *Not*I/*Pst*I clones analysed (11/12) showed a GC content of more than 50% (Table 1). Moreover, six clones showed a CpG ratio of more than 0.6. The other clones, except clone no. 1, also had a CpG ratio close to 0.6, and all of these clones, including clone no. 1, were located near CpG islands (data not shown). These results suggest that the fragments isolated by RLGS with *Not*I may have sequence characteristics related to those of CpG-island DNA. To identify potential gene regions associated with these CpG islands, we performed BLAST searches of nucleotide databases for

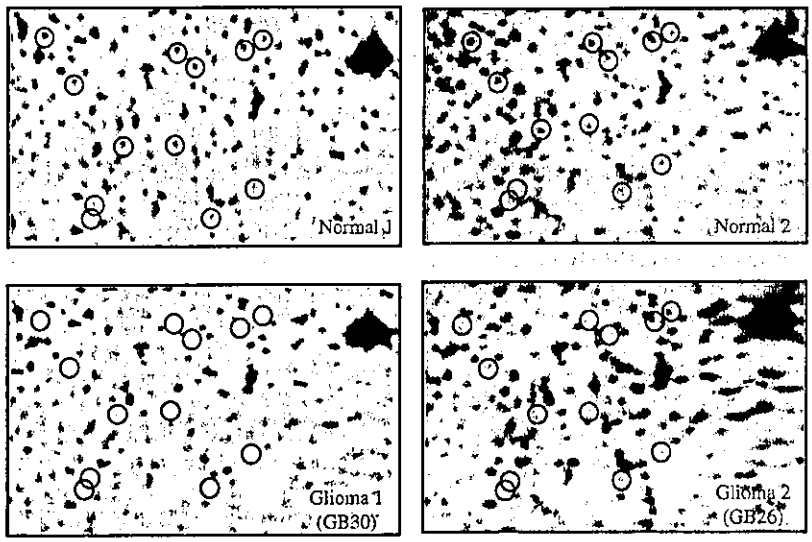


Figure 1 RLGS profile of human gliomas and matched normal lymphocytes. The intensity of the 12 spots was entirely lost or decreased in both the human glioma samples as compared with that in matched normal lymphocytes when RLGS was performed with the methylation-sensitive enzyme *Not*I

Table 1 Identification of each of the *Not*I/*Pst*I fragments and analysis of the GC content and CpG ratio

Clone no.	*Chromosome*	*GC%*	*CpG ratio*[a]	*Related gene*	*Location*	*Function of gene*
1	19	65	0.53	RFX1	Intron	Transcription regulator
2	15	54.4	0.77	None		
3	12	60.9	0.59	BGT-1	3′	Neurotransmitter transporter
4	Not identified					
5	5	63.8	0.67	ADAMTS2	Intron	Protease
6	2	64.6	0.94	HOXD9	5′	Transcription factor
7	9	63.8	0.59	None		
8	15	67.8	0.59	FKSG88	Exon and intron	Unknown
9	14	58.3	0.78	CGI-112 protein	5′	Unknown
10	X	67.3	0.61	None		
11	12	67.1	0.58	ALK-1	3′	Intracellular signal transducer
12	2	64.4	0.91	None		

[a]CpG ratio; {(number of CpGs)/(number of guanines)(number of cytosines)} (number of nucleotides analysed) of each clone

each of the 11 clones, and identified seven genes that may be aberrantly methylated in gliomas.

Analysis of gene expression by RT–PCR

To examine the relationship between CpG methylation and gene expression, we analysed the expression pattern of these seven identified genes by RT–PCR in a human normal brain tissue, glioma tissues, and glioma cell lines. The expressions of RFX1, BGT-1 (unpublished data), and HOXD9 (unpublished data) in the glioma specimens were significantly different from those in normal brain tissue. We focused on the analysis of RFX1 in this study, because expression of the RFX1 gene was found to be frequently decreased in human glioma cell lines (5/5) and tissues (6/9) (Figure 2). The PCR products were confirmed to be specific for the RFX1 gene by sequencing (data not shown).

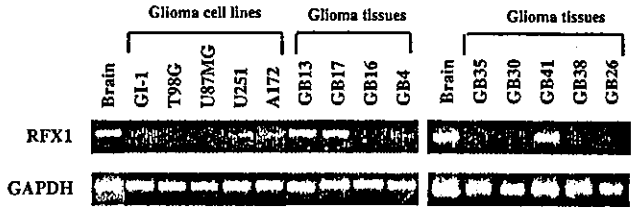


Figure 2 RT–PCR analysis of the RFX1 gene in human normal brain tissue and glioma cell lines/tissues. The expression of the RFX1 gene was decreased in glioma cell lines (5/5) and tissues (6/9) as compared with that in normal brain tissue

Methylation of a CpG island in intron 7 of the RFX1 gene

The BLAST search of nucleotide databases revealed that the isolated CpG island associated with the RFX1 gene was located in the seventh intron of the gene (Figure 3a). To examine the methylation status of the CpGs in the seventh intron of the RFX1 gene in detail, bisulfite sequencing was performed. As shown in Figure 3b, in the RFX1 gene region spanning intron 7, the entire regions of the bisulfite-PCR products in the glioma cell lines and tissues were densely methylated, whereas the corresponding CpG islands in normal peripheral blood mononuclear cells (PBMCs) and brains were hypomethylated. Thus, CpGs in the RFX1 region spanning intron 7 were confirmed to be hypermethylated in human gliomas. However, the CpG island in the 5′ UTR region (−473 to −32) of the RFX1 gene did not show any aberrant methylation in U251 glioma cells (data not shown).

Enhancer activity of intron 7 of the RFX1 gene

To examine the role of the RFX1 region spanning intron 7 in gene transcription, we analysed the enhancer activity of the seventh intron by reporter gene assay. The PCR-amplified intron 7 of the RFX1 gene, which is not methylated, was subcloned into the pGL3-Promoter vector downstream of the luciferase reporter gene driven by the SV40 promoter in both orientations. The reporter gene assay revealed that the positive control vector pGL3-Control, containing the SV40 promoter and enhancer, increased the expression of the luciferase reporter gene to approximately four times as much as that in the pGL3-Promoter vector containing the SV40 promoter only (Figure 4). The pGL3-Promoter vectors with the seventh intron of RFX1 in both orientations showed a 2- to 3.5-fold increase in the expression as compared with the pGL3-Promoter vector. These results suggest that the seventh intron of RFX1 has enhancer activity and the expression of RFX1 may be controlled by the seventh intron region in human gliomas.

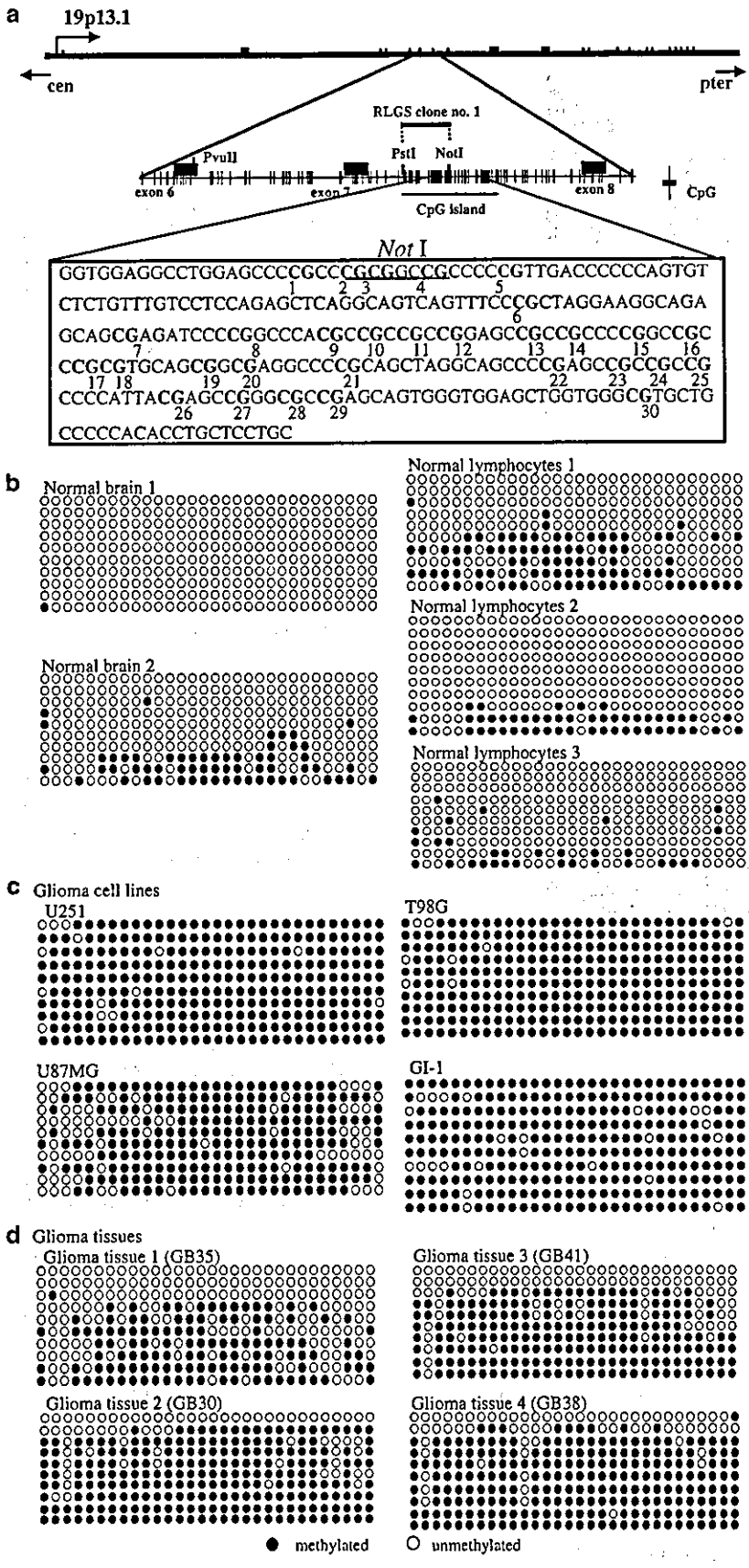
Re-expression of RFX1 in glioma cells by treatment with 5-azacytidine and/or trichostatin A

To examine the role of methylation and histone deacetylation in the silencing of RFX1 expression in human gliomas, we treated the human glioma cell line U251 with 5-azacytidine (5-aza-C), a methyltransferase inhibitor, and/or trichostatin A (TSA), a histone deacetylase inhibitor. RT–PCR analysis revealed that treatment with 5-aza-C induced an increase in RFX1 expression (Figure 5). Combined treatment with 5-aza-C and TSA resulted in a greater induction of RFX1 expression. These results suggest that DNA methylation may cause the silencing of RFX1 expression in human gliomas.

Cell growth suppression by RFX1

The expression of RFX1, which is a transcriptional repressor of the proto-oncogene c-*myc*, was shown to be frequently downregulated in human gliomas. To analyse the function of RFX1 in gliomas, we examined the relationship between RFX1 expression and the cellular proliferative activity in gliomas. Thymidine uptake of U251 glioma cells was measured 48 h after gene transfection for the analysis of cellular proliferative activity. Western blot analysis revealed that U251 glioma cells did not express the RFX1 protein (Figure 6a). The RFX1-transfected U251 cells showed the RFX1 protein expression (Figure 6a) and a significant inhibition of the cellular proliferative activity as compared with that of U251 cells or U251 cells transfected with the control vector (Figure 6b). These results suggest that RFX1 may be involved in the cellular proliferative activity in gliomas.

Figure 3 Analysis of CpG methylation. (a) Schematic representation of the genomic position of RLGS clone no.1 and the CpG island in intron 7 of the RFX1 gene. The black boxes show the exons of RFX1. A CpG plot was drawn using GENETYX-MAC, Ver.11 (Software Development Co. Ltd) with modification. In the sequence, CpG dinucleotides are shown in bold and are numbered below each site. (b) Methylation maps derived from bisulfite sequencing analysis of human glioma cell lines, glioma tissues, normal brains, and normal lymphocytes


a
19p13.1
cen
pter
RLGS clone no. 1
PvuII
PstI
NotI
exon 6
exon 7
exon 8
CpG
CpG island
Not I
GGTGGAGGCCTGGAGCCCCGCCCGCGGCCGCCCCCGTTGACCCCCCAGTGT
CTCTGTTTGTCCTCCAGAGCTCAGGCAGTCAGTTTCCCGCTAGGAAGGCAGA
GCAGCGAGATCCCCGGCCCACGCCGCCGCCGGAGCCGCCGCCCCGGCCGC
CCGCGTGCAGCGGCGAGGCCCCGCAGCTAGGCAGCCCCGAGCCGCCGCCG
CCCCATTACGAGCCGGGCGCCGAGCAGTGGGTGGAGCTGGTGGGCGTGCTG
CCCCCACACCTGCTCCTGC
b
Normal brain 1
Normal brain 2
Normal lymphocytes 1
Normal lymphocytes 2
Normal lymphocytes 3
c
Glioma cell lines
U251
T98G
U87MG
GI-1
d
Glioma tissues
Glioma tissue 1 (GB35)
Glioma tissue 2 (GB30)
Glioma tissue 3 (GB41)
Glioma tissue 4 (GB38)
methylated
unmethylated

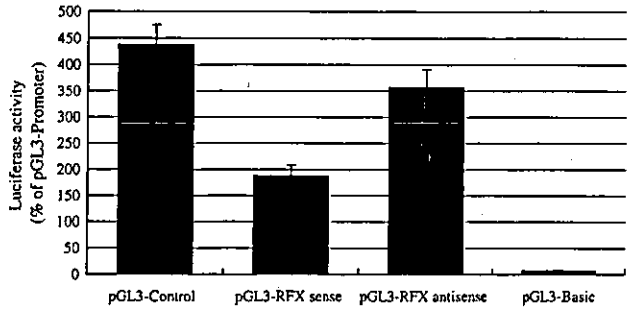


Figure 4 Analysis of the enhancer activity of intron 7 of the RFX1 gene. pGL3-Promoter vector contains the SV40 promoter upstream of the luciferase reporter gene. The region of the seventh intron of the RFX1 gene was subcloned into the pGL3-Promoter vector downstream of the luciferase gene in both sense (pGL3-RFX sense) and antisense (pGL3-RFX antisense) orientations. pGL3-Control vector, which contains the SV40 promoter and enhancer sequences, was used as a positive control vector. pGL3-Basic vector, which lacks eucaryotic promoter and enhancer sequences, was used as a negative control vector. The seventh intron of the RFX1 gene enhanced the expression of the reporter gene driven by the SV40 promoter. The values indicate the luciferase activities compared to that obtained in the pGL3-Promoter vector containing the SV40 promoter alone (% of pGL3-Promoter)

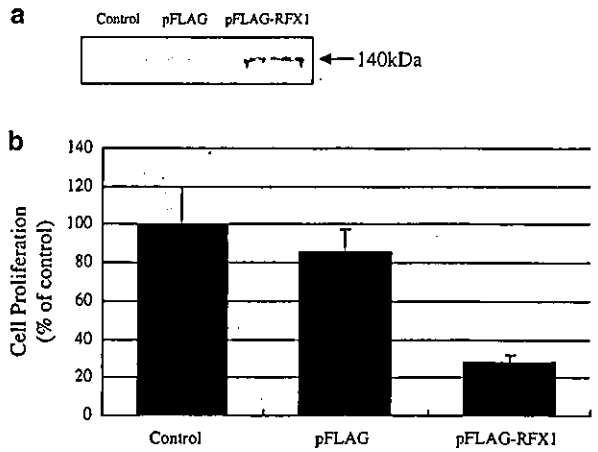


Figure 6 Suppression of the proliferative activity of glioma cells by transient expression of the RFX1 gene. (a) Western blot analysis of RFX1. RFX1 was not expressed in the control U251 cells or U251 cells transfected with the control vector pFLAG. The RFX1 protein was detected in cells transfected with pFLAG-RFX1 using an anti-RFX1 polyclonal antibody. (b) [^{3}H]thymidine uptake assay in U251 human glioma cells. The proliferation of RFX1-expressing U251 glioma cells was significantly reduced as compared with that of control U251 glioma cells or U251 cells transfected with the control vector pFLAG

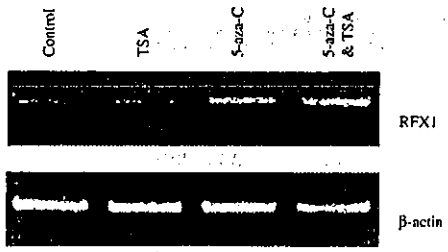


Figure 5 Re-expression of RFX1 after treatment with a methyltransferase, 5-aza-C, and/or a histone deacetylase inhibitor, TSA. RT–PCR analysis of the RFX1 gene was performed after the treatment of U251 human glioma cells with 5-aza-C, TSA, or both 5-aza-C and TSA

Discussion

In this study, we analysed the methylation status of CpG islands associated with tumorigenesis in glioma specimens by RLGS with a CpG methylation-sensitive enzyme, *Not*I, and potential gene regions associated with the CpG islands were identified. When RLGS is performed with a CpG methylation-sensitive enzyme, alterations in RLGS spots indicate several types of genomic changes. Loss or appearance of a spot may indicate deletion or mutation of genomic DNA, as well as DNA methylation or demethylation. An increase in the intensity of a spot may mean gene amplification or demethylation of another allele, whereas a decrease in the intensity may indicate allelic changes or methylation of one allele. In this study, all of the 11 spots isolated from approximately the 300 RLGS spots analysed, the intensity of which was either entirely lost or decreased in gliomas as compared with that in normal lymphocytes, possessed the sequence characteristics of CpG islands (Table 1). Sequence analysis revealed that no mutation or deletion was present in any of the DNA fragments isolated from the glioma samples (data not shown). More than 80% of the *Not*I sites lie in CpG islands (Lindsay and Bird, 1987). A recent report indicated that aberrant CpG-island methylation in cancer takes a tumor-type-specific pattern, and that significant preferential methylation of CpG islands was observed in gliomas (Costello *et al*., 2000). In addition, Costello *et al*. also performed an RLGS analysis with *Not*I of glioma in combination with an analysis of high-resolution deletion maps from microarray-based comparative genomic hybridization, and the results suggested that aberrant CpG-island methylation rarely occurs together with deletions (Zardo *et al*., 2002). In our study, three of the 11 CpG islands (clones 1, 3, and 6) were confirmed to be aberrantly methylated in gliomas by bisulfite sequencing (unpublished data). Taken together, our results suggest that RLGS with the *Not*I enzyme is a useful method to analyse the methylation status of CpG islands, and aberrant methylation of CpG islands was found to occur frequently in gliomas.

Methylation of CpGs within the promoter region of genes is often associated with transcriptional inactivation. The expression analysis of the identified genes related with the methylated CpG islands revealed that the expression of the transcriptional regulator RFX1 was decreased in glioma tissues and cell lines as compared with that in normal brain tissue (Figure 2).

To evaluate whether the transcriptional inactivation of the RFX1 gene was due to DNA methylation, we studied the effect of the DNA methyltransferase inhibitor 5-aza-C on the expression of the RFX1 gene in glioma cells. Treatment with 5-aza-C induced re-expression of RFX1 in the glioma cells, suggesting that DNA methylation may suppress transcription of the RFX1 gene. In addition, treatment with the histone deacetylase inhibitor TSA concomitantly with 5-aza-C induced even greater expression of RFX1, suggesting that deacetylation of histones may also play a role in the suppression of transcription of the RFX1 gene.

Regarding the region of DNA methylation, while the seventh intron was hypermethylated in all the glioma cell lines and tissues analysed, no aberrant methylation was detected in the promoter region of the RFX1 gene in glioma cells. The reporter gene assay revealed that the seventh intron has enhancer activity in relation to gene transcription. Transcriptional repression of the Keratin 18 gene is due to the methylation of a CpG in the intron enhancer element (Umezawa *et al.*, 1997). Methylated DNA-binding proteins, such as MeCP-2, bind DNA with a single CpG methylation, and can inhibit gene transcription (Meehan *et al.*, 1992). Although it is necessary to further analyse the mechanisms of the gene transcription of RFX1, differential methylation of CpGs within the enhancer element may play an active role in suppressing the expression of RFX1 in gliomas.

RFX1 belongs to the family of genes having an evolutionarily conserved DNA-binding domain named the winged helix (Emery *et al.*, 1996; Gajiwala *et al.*, 2000). RFX1 binds and regulates the enhancers of a number of virus and cellular genes, including the proto-oncogene c-*myc* (Chen *et al.*, 2000; Katan-Khaykovich and Shaul, 2001). The expression of c-*myc* is repressed by the binding of RFX1 to the intron 1 element. RT–PCR analysis revealed that RFX1 expression is frequently decreased in human glioma tissues and glioma cell lines. Moreover, re-expression of RFX1 in glioma cells by gene transfection caused inhibition of the cellular proliferative activity. Therefore, it is considered that RFX1 may be involved in glioma tumorigenesis as a tumor suppressor.

Materials and methods

Tumor samples and cell lines

Tumor tissues (glioblastoma, GB4, GB13, GB16, GB17, GB38, and GB41; anaplastic astrocytoma, GB35; anaplastic oligoastrocytoma, GB30; and mixed glioma (WHO grade II), GB26) and adjacent non-neoplastic brain tissue specimens were obtained from glioma patients who were operated on at the Department of Neurosurgery, Keio University School of Medicine. All the samples were frozen in liquid nitrogen immediately after collection and stored at −80°C until preparation of the DNA samples. Written informed consent for the study conducted in conformity with the Institutional Review Board guidelines was granted by all of the patients. The human glioma cell lines, T98G, U87MG, and A172, obtained from the American Type Culture Collection, and U251 and GI-1, obtained from the RIKEN GENE BANK, were cultured in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (JRH Biosciences, USA).

RLGS profile analysis

RLGS was performed according to published protocols (Hatada *et al.*, 1991). Briefly, high-molecular-weight genomic DNA isolated from tumor tissues and PBMCs (Blin and Stafford, 1976) was digested with *Not*I and *Pvu*II restriction enzymes, and the *Not*I-derived 5′ protruding ends were ^{32}P-labeled. After first-dimension separation of the ^{32}P-labeled DNA fragments in agarose disc gels, a second digestion was conducted *in situ* using *Pst*I. The resulting DNA fragments were separated perpendicularly on a polyacrylamide gel. The separated DNA fragments were visualized by autoradiography. The RLGS profiles were digitized on a VXR-8 Digitizer (Vidar Systems Corporation, Hendon, VA, USA) and the positions and intensities of the spots were analysed on a Macintosh computer (System 9.2; Apple Computer Inc., CA, USA) with Adobe Photoshop 5.0J (Adobe Systems Incorporated, CA, USA).

Isolation and sequence analysis of the DNA fragments on the RLGS profile

The DNA fragments on the RLGS profile were electro-eluted and purified by phenol/chloroform/isoamyl alcohol extraction, followed by ethanol precipitation. The eluted DNAs initially ligated the *Not*I and *Pst*I adapters. The ligated fragments were amplified by PCR using a primer set with specific sequences for each adapter. The PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The sequencing reactions were performed with the BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Warrington, UK). BLAST searches of the resultant sequences were performed in the databases provided by the National Center for Biotechnology Information (NCBI).

RT–PCR analysis

Total RNAs were isolated from human glioma tissues and cell lines using Trizol (Gibco BRL). Total RNA from normal brain tissue was purchased from BD Biosciences Clontech. Synthesis of cDNA was performed on 10 μg from total RNA using reverse transcriptase XL (AMV) (Takara, Tokyo, Japan), and transcribed in a final volume of 100 μl. Using Takara Taq™ (Takara, Tokyo, Japan), 1 μl of cDNA template was amplified in a 25-μl reaction mixture containing 0.1 μM of each primer. The primer combinations were as follows: RFX1, sense 5′-GAA GAT GGA AGG CAT GAC C-3′ and antisense 5′-GGC TCT TGG CAA AGT TCC-3′; GAPDH, sense 5′-TGA ACG GGA AGC TCA CTG G-3′ and antisense 5′-TCC ACC ACC CTG TTG CTG TA-3′; β-actin, sense 5′-GTC GAC AAC GGC TCC GGC ATG TGC-3′ and antisense 5′-GGA TCT TCA TGA GGT AGT CAG TCA G-3′. The PCR conditions were as follows: RFX1, 94°C 5 min, 94°C 1 min, 57°C 1 min, and 72°C 1 min over 30 cycles; GAPDH, 94°C 5 min, 94°C 1 min, 58°C 1 min, and 72°C 1 min over 25 cycles; and β-actin, 94°C 5 min, 94°C 1 min, 68°C 1 min, 72°C 2 min over 20 cycles.

Bisulfite genomic sequencing

Bisulfite conversion was performed with 1 μg of genomic DNA and the reagents provided in the Intergen's CpGenome DNA