In conclusion, visceral obesity was the independent risk factor for the incidence of PPCs after pancreaticoduodenectomy. Preoperative VFA measurements using CT scan may be a useful tool for the prediction of the development of PPCs compared to BMI calculation and may reduce the incidence of PPCs through careful management of patients with high VFA.

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ORIGINAL ARTICLE

Predictive risk factors for clinically relevant pancreatic fistula analyzed in 1,239 patients with pancreaticoduodenectomy: multicenter data collection as a project study of pancreatic surgery by the Japanese Society of Hepato-Biliary-Pancreatic Surgery

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Abstract

Background/purpose It is important to predict the development of clinically relevant pancreatic fistula (grade B/C) in the early period after pancreaticoduodenectomy (PD). This study has been carried out as a project study of the Japanese Society of Hepato-Biliary-Pancreatic Surgery (JSHPBS) to evaluate the predictive factors associated with clinically relevant pancreatic fistula (grade B/C).

Method The data of 1,239 patients from 11 medical institutions who had undergone PD between July 2005 and June 2009 were retrospectively analyzed to review patient characteristics and perioperative and postoperative parameters.

Results A drain amylase level >4,000 IU/L on postoperative day (POD) 1 was proposed as the cut-off level to predict clinical relevant pancreatic fistula by the receiver operating characteristic (ROC) curve. The sensitivity, specificity, and accuracy of this cut-off level were 62.2, 89.0, and 84.8%, respectively. A multivariate logistic regression analysis revealed that male [odds ratio (OR) 1.7,

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$P = 0.039$], intraoperative bleeding >1,000 ml (OR 2.5, $P = 0.001$), soft pancreas (OR 2.7, $P = 0.001$), and drain amylase level on POD 1 >4,000 IU/L (OR 8.6, $P < 0.001$) were the significant predictive factors for clinical pancreatic fistula.

Conclusion The four predictive risk factors identified here can provide useful information useful for tailoring postoperative management of clinically relevant pancreatic fistula (grade B/C).

Keywords Pancreatic fistula · Pancreaticoduodenectomy · Predictive risk factors · Drain amylase level

Introduction

In most patient series, the incidence of pancreatic fistula has been reported to vary between 5 and 20% after pancreaticoduodenectomy (PD) and to be associated with a high mortality rate [1–7]. In 2005, the International Study Group of Pancreatic Fistula (ISGPF) proposed a consensus definition and clinical grading of postoperative pancreatic fistula [8]. The most important issue currently being debated regarding pancreatic fistulas is whether it is possible to predict the development of clinically relevant pancreatic fistula (grade B/C) according to the ISGPF proposal in the early period after PD. The risk factors for developing a pancreatic fistula in previously reported studies may have been able to predict all grades of pancreatic fistulas but, unfortunately, could not predict the extent of severe clinically relevant pancreatic fistula (grade B/C) [9–13].

Molinari et al. [14] proposed that a drain amylase value on postoperative day (POD) 1 of >5,000 U/L was a significant predictive factor for the incidence of all grades of pancreatic fistula after PD. However, this amylase value could not distinguish clinically relevant pancreatic fistulas (grade B/C) from insignificant disease during the early postoperative period. Kawai et al. proposed that a combination of two predictive postoperative factors on POD 4, namely, serum albumin level ≤3.0 g/dL and leukocyte counts >9,800/mm^3, can predict the development of clinically relevant pancreatic fistula [15]. However, these two factors may also reflect serious systemic inflammation, such as that due to other intra-abdominal or respiratory complications. It therefore remains unclear which predictive risk factor(s) can be used to precisely distinguish the risk of clinically relevant pancreatic fistula (grade B/C) in the early postoperative period. In an attempt to clarify this situation, the Japanese Society of Hepato-Biliary-Pancreatic Surgery (JSHPBS) decided to perform a survey of high-volume PD centers in Japan to evaluate the predictive factors for the development of clinically relevant pancreatic fistula (grade B/C) in the early period after PD.

Methods

Patients

Data were collected by a questionnaire survey on all patients who underwent PD between July 2005 and June 2009 at one of 11 high-volume centers participating in the project study of the JSHPBS. The following patient characteristics and perioperative and postoperative parameters were reviewed: age, gender, body mass index (BMI), American Society of Anesthesiologists (ASA) class, preoperative laboratory data, such as hemoglobin, creatinine, HbA1c (glycated hemoglobin), albumin, total bilirubin, and amylase, preoperative biliary drainage, length of the surgery, intraoperative bleeding, blood transfusion, pancreatic texture (soft or hard), presence or absence of dilatation of the main pancreatic duct, histologic diagnosis (malignant or benign), and the serum C-reactive protein (CPR) and drain amylase levels on POD 1, 3, and 4. In total, data on 1,331 patients were collected from the 11 institutions. Of these 1,331 patients, 1,239 (749 men, 490 women; median age 67 years, age range 35–91 years) were enrolled in the study, and their data used for the analysis of the occurrence of pancreatic fistula using the ISGPF criteria.

Postoperative complications

The diagnosis of pancreatic fistula was made based on the ISGPF guidelines [8], namely, an amylase level in the drainage fluid on POD 3 of more than threefold the serum amylase level. Pancreatic fistulas were classified into three categories according to the ISGPF guidelines:

> Grade A: Transient fistula. There is no clinical impact. The patient is fed orally and remains clinically well.
> Grade B: Patients are usually supported with partial, total parenteral, or enteral nutrition. Antibiotics are usually used for signs of infections and a somatostatin analogue may also be required. Percutaneous drainage or persistent drainage for more than 3 weeks is usually required.
> Grade C: A major change in clinical management or deviation from the normal clinical pathway. Total parenteral, enteral nutrition, antibiotics, or somatostatin analogue is often instituted in an intensive care unit (ICU) setting. Radiologic intervention or reoperation is required. The patients typically require an extended hospital stay with a major delay in hospital discharge and have life-threatening complications, such as intra-abdominal bleeding or sepsis. There is a real possibility of postoperative mortality[8].

Grades B + C were defined as "clinically relevant pancreatic fistula". Delayed gastric emptying (DGE) was

defined according to a consensus definition and the clinical grading of postoperative DGE according to the proposals of the International Study Group of Pancreatic Surgery (ISGPS) [16], using the web-based calculator (http://pancreasclub.com/calculator/) to improve the homogeneity of the definition [17]. DGE was then classified into three categories (grade A, B, or C) by the ISGPS clinical criteria based on the clinical course and postoperative management, such as reinsertion of a nasogastric tube, the period of inability to tolerate a solid diet, presence or absence of vomiting, and the use of prokinetics. Other postoperative complications were graded according to the Clavien classification [18], where grade I is any deviation from the normal postoperative course but without any need for pharmacologic treatment or surgical, endoscopic, or radiologic intervention; grade II is indicated by complications requiring pharmacologic treatment; grade III, by complications requiring surgical, endoscopic, or radiologic intervention; grade IV, life-threatening complications requiring intermediate or ICU management; grade V, death. Complications in this study were defined as a condition that was more than grade II according to the Clavien classification.

Statistical analysis

Continuous variables were expressed as the mean ± standard deviation (SD). Patient characteristics and perioperative and postoperative factors between the groups were compared using chi-square statistics, the Fisher exact test, and the Mann–Whitney U test. Variables with $P < 0.05$ were entered into a logistic regression model to determine independent risk factors of postoperative complications. The independent risk factors of the variables were expressed as odds ratios (OR) with their 95% confidence intervals (CI). The measurement of drain amylase levels on POD 1 has a major benefit by enabling the development of clinically relevant pancreatic fistula (grade B/C) to be predicted in the early period after PD. In fact, amylase values in drains on POD 1 of >5,000 U/L have been reported to be a significant predictive factor for the incidence of all grades of pancreatic fistula after PD [14]. Therefore, the optimal cut-off levels of the drain amylase level on POD 1 for differentiation between the no pancreatic fistula/grade A group and the grade B/C group were sought by constructing receiver operating characteristic (ROC) curves, which were generated by calculating the sensitivities and specificities of the drain amylase level on POD 1 at several predetermined cut-off points. Line graphs were used for graphical visualization (SPSS, Chicago, IL). Statistical significance was defined as $P < 0.05$.

Results

The indications for PD in the 1,239 patients were 573 pancreatic adenocarcinoma, 237 bile duct carcinoma, 124 ampullary adenocarcinoma, 127 intraductal papillary neoplasms, 38 duodenal adenocarcinoma, 37 pancreatic endocrine neoplasms, 46 tumor-forming pancreatitis, and 57 "other" diseases.

Postoperative complications

Table 1 shows the postoperative complications among the patient cohort after PD. The overall morbidity was 44.2% (548/1,239 patients). The overall rate of pancreatic fistula was 30.2% (374 patients). When the pancreatic fistula was classified into the three categories according to the ISGPF criteria, 15.8% (196/1,239) of the patients had grade A fistula; 11.8% (146 patients) had grade B; 2.6% (32 patients) had grade C. In total 139 (11.2%) patients had intra-abdominal abscess, and 58 patients (4.7%) required percutaneous drainage for the development of intra-abdominal abscess related to pancreatic fistula after PD. The reoperation rate due to pancreatic fistula was 0.72% (9/1,239 patients), and the overall incidence of DGE was 16.9% (211/1,239 patients). The DGE was categorized according to the ISGPS guidelines into grade A (107 patients, 9.6%), grade B (51 patients, 4.1%), and grade C (53 patients, 4.3%). The overall mortality rate was 0.97% (12/1,239 patients).

Comparison of patient characteristics, intraoperative status, and postoperative outcome among types of pancreatic fistula

Table 2 shows the general characteristics, intraoperative status, and postoperative outcome of the 1,239 patients classified by ISGPF. Based on the ISGPF criteria, 865 of the 1,239 patients (69.8%) did not develop a pancreatic fistula, and 196 patients (15.8%) developed only transient pancreatic fistula (grade A), while a clinically relevant pancreatic fistula (grade B/C) developed in 178 patients (14.4%). The ratio of male to female patients was significantly higher in those patients with a grade B/C fistula than in those with no pancreatic fistula or a grade A fistula. In those patients with a grade B/C fistula, the length of the surgery was significantly longer, and the intraoperative bleeding or the need for blood transfusion was significantly higher, compared to subjects with no pancreatic fistula or a grade A fistula. Patients with soft pancreatic parenchyma or a pancreatic duct <3 mm had a significantly higher incidence of pancreatic fistula (grade A and grade B/C) than those with no pancreatic fistula ($P < 0.01$). There were no

Table 1 Postoperative complications and the outcome after 1,239 pancreaticoduodenectomies

Postoperative complications/outcome	No. of patients (%)
Overall morbidity	548 (44.2)
Local complication	
Pancreatic fistula[a]	374 (30.2)
Grade A	196 (15.8)
Grade B	146 (11.8)
Grade C	32 (2.6)
Intra-abdominal abscess	139 (11.2)
Intra-abdominal bleeding	34 (2.7)
Biliary leakage	29 (2.3)
Gastrointesitinal leakage	72 (5.8)
Delayed gastric emptying[b]	211 (16.9)
Grade A	107 (9.6)
Grade B	51 (4.1)
Grade C	53 (4.3)
Wound infection	130 (10.5)
Systemic complications	
Cardiac complication	35 (2.8)
Pulmonary complications	23 (1.9)
Sepsis	35 (2.8)
Percutaneous drainage	58 (4.7)
Persisting drain (more than 3 weeks)[c]	104 (8.4)
Reoperation	9 (0.7)
Mortality	12 (1.0)

[a] Pancreatic fistula as defined by the International Study Group of Pancreatic Fistula (ISGPF)

[b] Delayed gastric emptying as defined by the International Study Group of Pancreatic Surgery (ISGPS)

[c] Used in postoperative management of intra-abdominal abscess related to pancreatic fistula

significant differences between patients with grade A and grade B/C pancreatic fistual in term of the presence of soft pancreatic parenchyma or pancreatic duct <3 mm.

With regard to the postoperative outcome, the drain amylase level on POD 1 in the grade B/C group (18,846 ± 37,287 IU/L) was significantly higher than that in the grade A group (7,951 ± 15,322 IU/L) ($P = 0.002$), whereas there was no significant difference between the grade A group and grade B/C group in terms of drain amylase level on POD 3 and 4.

Predictive factors for the development of a clinically relevant pancreatic fistula (grade B/C)

With regard to the sensitivity and specificity of the drain amylase level on POD 1, an area under the ROC curve of 0.840 was obtained ($P < 0.001$; 95% confidence interval: 0.807–0.874) (Fig. 1). Drain amylase level >4,000 IU/l on POD 1 was suggested to be the best cut-off for prediction of the clinically relevant pancreatic fistula. The sensitivity, specificity, accuracy, positive predictive value and negative predictive value of drain amylase level on POD1 >4,000 IU/l were 62.2, 89.0, 84.8, 51.1 and 92.7%, respectively.

Univariate and multivariate analysis were used to reveal those factors predicting grade B/C pancreatic fistula. Table 3 presents the results of the univariate analysis of 18 parameters as potential risk factors in the 178 patients with clinically relevant pancreatic fistula (grade B/C) versus 1,061 patients with no pancreatic fistula or with transient pancreatic fistula (grade A). Nine factors were extracted and identified as being useful for discriminating between those who would develop grade B/C fistula and those who would not develop a fistula or would develop only grade A fistula: (1) preoperative factors, namely, male gender ($P < 0.001$), BMI >25 kg/m^2 ($P = 0.005$), HbA1c >7.0% ($P = 0.001$), and creatinine >1.5 mg/dL ($P = 0.039$); (2) intraoperative factors, namely, operative time >480 min ($P = 0.012$), intraoperative bleeding >1,000 mL ($P = 0.001$), soft pancreatic parenchyma ($P < 0.001$), and main pancreatic duct <3 mm ($P < 0.001$); (3) one postoperative factor, namely, drain amylase level on POD 1 >4,000 IU/L ($P < 0.001$). A multivariate logistic regression analysis revealed that male gender (OR 1.7, 95% CI 1.0–3.0, $P = 0.039$), intraoperative bleeding >1,000 mL (OR 2.5, 95% CI 1.4–4.6, $P = 0.001$), soft pancreatic parenchyma (OR 2.7, 95% CI 1.5–4.8, $P = 0.001$), and drain amylase level on POD 1 >4,000 IU/L (OR 8.6, 95% CI 5.2–14.2, $P < 0.001$) were the significant predictive factors for developing clinically relevant pancreatic fistula of grade B/C (Table 4).

Discussion

The development of pancreatic fistula has been reported to be a potentially life-threatening complication after PD [19–21]. Although lower grade fistula can still complicate patient recovery, it is very important to be able to predict whether a patient will develop clinically relevant pancreatic fistula (grade B/C) in the early period after PD, since these fistulas require changes in patient management and are associated with a higher mortality rate. This study was conducted as a project study of the JSHPBS and was designed to evaluate the predictive factors associated with the development of a clinically relevant pancreatic fistula (grade B/C) after PD. We found that male gender, intraoperative bleeding >1,000 mL, soft pancreatic parenchyma, and drain amylase level on POD 1 of >4,000 IU/L were the most significant predictive factors of the development of clinically relevant pancreatic fistula (grade B/C).

Table 2 Comparison of patient characteristics, intraoperative status, and postoperative outcome among types of pancreatic fistula

Patient characteristics, intraoperative status, and postoperative outcome	Pancreatic fistula		
	(−) (n = 865)	Grade A (n = 196)	Grade B/C (n = 178)
Patient characteristics			
Age (years)	66 ± 11	67 ± 10	67 ± 9
Gender (male/female)	507/358	110/86	132/46[c]
Body mass index (kg/m^2)	21.5 ± 3.4	21.7 ± 3.0	22.6 ± 3.3
ASA (I–II/III–IV)	740/122	172/24	144/33
Preoperative serum bilirubin level (mg/dl)	3.1 ± 4.6	2.9 ± 4.5	3.4 ± 5.9
Preoperative biliary drainage (%)	46.2	41.8	49.4
Preoperative hemoglobin (g/dL)	12.4 ± 1.6	12.3 ± 1.8	12.7 ± 1.7
Preoperative creatinine (mg/dL)	0.8 ± 10.6	0.8 ± 0.5	1.0 ± 1.0
HbA1c (%)	6.3 ± 4.5	5.8 ± 1.3	5.5 ± 0.9
Preoperative serum albumin (g/dL)	3.9 ± 0.5	3.9 ± 0.5	3.8 ± 0.5
Preoperative serum amylase (IU/L)	139 ± 244	137 ± 162	125 ± 113
Histology (benign/malignant)	132/696	43/144	35/132
Intraoperative status			
Operative time (min)	480 ± 144	456 ± 133	542 ± 403[c]
Intraoperative bleeding (mL)	1,215 ± 1,168	1,170 ± 1,056	1,596 ± 1,849[c]
Red blood cell transfusion (unit)	1.8 ± 3.9	1.7 ± 2.8	2.9 ± 6.2[c]
Pancreatic duct (>3 mm/<3 mm)	573/170	99/77[a]	76/90[b]
Soft pancreatic texture (soft/hard)	355/510	151/45[a]	142/36[b]
Postoperative outcome			
CRP on POD 1 (mg/dL)	9.1 ± 22.0	8.6 ± 3.3	9.3 ± 3.8
CRP on POD 3 (mg/dL)	12.0 ± 5.95	15.8 ± 7.4[a]	20.0 ± 7.2[c]
CRP on POD 4 (mg/dL)	8.35 ± 5.95	12.2 ± 6.1[a]	15.7 ± 6.7[c]
WBC on POD 1 (/mm^3)	10,167 ± 3,367	10,636 ± 3,757	10,380 ± 4,163
WBC on POD 3 (/mm^3)	9,127 ± 5,024	10,489 ± 3,562[a]	10,674 ± 3,840[b]
WBC on POD 4 (/mm^3)	7,677 ± 6,169	8,799 ± 3,168[a]	9,735 ± 3,529[b]
Albumin on POD 1 (g/dL)	2.8 ± 1.0	2.9 ± 0.5	2.9 ± 0.5
Albumin on POD 3 (g/dL)	2.8 ± 0.5	2.8 ± 0.5	2.7 ± 0.5
Albumin on POD 4 (g/dL)	2.9 ± 0.6	3.0 ± 0.6	2.7 ± 0.4[b]
Amylase level of drainage fluid			
POD 1 (IU/L)	1,058 ± 2,186	7,951 ± 15,322[a]	18,846 ± 37,278[c]
POD 3 (IU/L)	134 ± 181	3,638 ± 10,711[a]	6,284 ± 15,183[c]
POD 4 (IU/L)	79 ± 136	2,375 ± 9,565[a]	5,325 ± 18,452[c]
Postoperative hospital stay (days)	28 ± 18	29 ± 20	56 ± 40[c]

Data are given as the mean ± standard deviation (SD) unless otherwise indicated

ASA American Society of Anesthesiologists, *HbA1c* hemoglobin A1C (glycated hemoglobin), *CRP* C-reactive protein, *WBC* white blood cells, *POD* postoperative day

[a] No pancreatic fistula vs. grade A, $P < 0.01$

[b] No pancreatic fistula vs. grade B + C, $P < 0.01$

[c] No pancreatic fistula vs. grade B + C, $P < 0.01$ and grade A vs, grade B + C, $P < 0.01$

In particular, a drain amylase value >4,000 U/L on POD 1 correlated with 8.6-fold increased risk of developing clinically relevant pancreatic fistula.

Some authors have proposed that drain-related data, including the drain amylase level, are useful for defining the risk of developing a pancreatic fistula after PD [14, 22, 23], including Molinari et al. [14], who suggested that a drain amylase value on POD 1 >5,000 U/L was a significant predictive factor for the incidence of pancreatic fistula. However, the latter study was limited due to the small sample

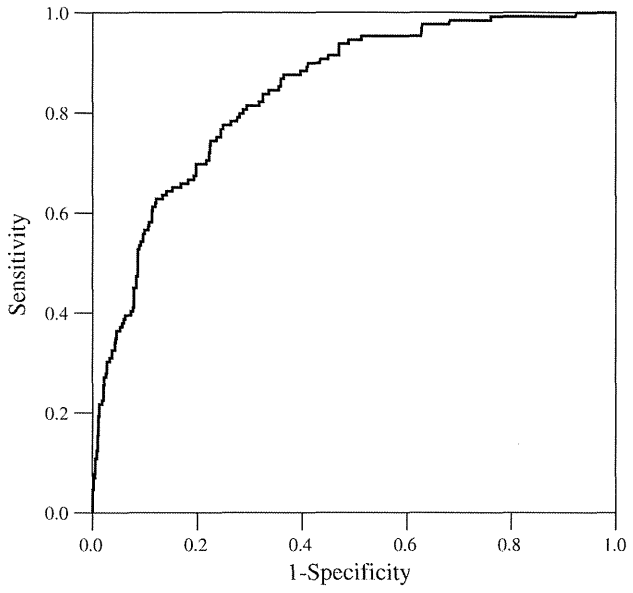

Fig. 1 Receiver operating characteristic (ROC) curves fore drain amylase level on postoperative day (POD 1) to predict clinically relevant pancreatic fistula (grade B/C). An area under the ROC curve of 0.841 was obtained taking the sensitivity and specificity of the drain amylase level on POD 1 into consideration [$P < 0.001$, 95% confidence interval (CI) 0.806–0.876]. A drain amylase level >4,000 IU/l on POD 1 was therefore suggested to be the best cut-off for predicting the clinical occurrence pancreatic fistula

size ($n = 137$). Moreover, of the 137 patients enrolled in the study, 36 patients underwent distal pancreatectomy, and 12 (44%) of the 27 patients with pancreatic fistula reported in their results underwent distal pancreatectomy, not PD [14]. In these three studies [14, 22, 23], clinically relevant pancreatic fistula (grade B/C) could not be distinguished from transient (grade A) fistula in the early postoperative period solely based on the drain amylase value. In three other studies [15, 24, 25], the authors were unable to determine whether the drain amylase value was reflective of clinically relevant pancreatic fistula, although they did propose the evaluation of predictive risk factors, such as the presence of soft pancreas, intra-abdominal bleeding, and the postoperative albumin level, as indicators for the development of clinically relevant pancreatic fistula. To the best of our knowledge, our study is the first published report demonstrating that the drain amylase value on POD 1 can predict the development of clinically relevant pancreatic fistula (grade B/C). Therefore, based on the results of this study, early removal of drains can be done when the drain amylase value is <4,000 U/L on POD 1 because these patients are unlikely to develop clinically relevant fistula. Moreover, early drain removal has previously been demonstrated to play a critical role in reducing the incidence of pancreatic fistula or intra-abdominal abscess [26, 27].

Table 3 A univariate analysis of the predictive factors for clinical relevant pancreatic fistula (ISGPF grade B/C)

Predictive factors	Pancreatic fistula grade B/C		
	(−) ($n = 1,061$)	(+) ($n = 178$)	P value
Age (>75/≤75 years)	202/859	31/147	0.608
Gender (male/female)	617/444	132/46	<0.001
BMI (>25/≤25 kg/m^2)	142/919	38/140	0.005
ASA (I–II/III–IV)	146/912	33/144	0.090
COPD (yes/no)	59/1,001	11/167	0.871
HbA1c (>7.0/≤7.0%)	181/743	15/129	0.008
Hemoglobin (>12/≤12 g/dL)	647/410	120/58	0.114
Creatinine (>1.2/≤1.25 mg/dL)	32/975	11/162	0.039
Albumin (>3.5/≤3.5 g/dL)	857/198	139/37	0.539
Total bilirubin (>5/≤5 mg/dL)	212/846	36/142	0.776
Amylase (>180/≤180 IU/L)	186/865	26/152	0.257
Preoperative biliary drainage (yes/no)	482/579	88/90	0.321
Operation time (>480/≤480 min)	470/591	97/81	0.012
Intraoperative bleeding (>1,000/≤1,000 mL)	478/583	105/73	0.001
Blood transfusion (yes/no)	344/717	69/109	0.097
Pancreatic texture (soft/hard)	506/555	142/36	<0.001
Main pancreatic duct (<3/≥3 mm)	247/672	90/76	<0.001
Amylase level of drainage fluid on POD 1 (IU/L) (≤4,000/>4,000)	712/88	56/92	<0.001

BMI body mass index, *COPD* chronic obstructive pulmonary disease

Table 4 Multivariate analysis of the predictive factors for clinical relevant pancreatic fistula (ISGPF grade B/C) and predictive score

Risk factor	*P* value	Odds ratio	95% Confidence interval
Male	0.039	1.7	1.0–3.0
Intraoperative bleeding >1,000 ml	0.001	2.5	1.5–4.2
Soft pancreas	0.001	2.7	1.5–4.8
Amylase level of drainage fluid on POD 1 (IU/l) >4,000 IU/L	<0.0001	8.6	5.2–14.2

In contrast, the authors of a number of other studies have found that the amylase level in drainage fluid after PD has no clinical significance [28, 29]. The ISGPF has proposed that the ability to detect pancreatic fistula is imperfect when only drain data are used [28]. Two factors may underlie this low predictive ability: first, there have been patients who ultimately demonstrated no clinically relevant symptoms of pancreatic fistula despite having amylase-rich fluid on POD 1; second, there was the pattern of pancreatic fistula defined by Pratt et al. [30] as latent pancreatic fistula. Latent fistulas have been defined as initially lacking amylase-rich fluid but ultimately becoming clinically relevant pancreatic fistula (grade B/C). Pratt et al. [30] proposed that advanced age and small pancreatic duct size were significantly associated with latent fistulas.

There are several limitations to our study because of the multicenter and retrospective nature of the data collection. First, the surgical procedures, such as pancreaticoenterostomy (pancreaticogastrostomy or pancreaticojejunostomy) or use of pancreatic duct stent (internal, external, or no stent), were not standardized across institutions. Second, drain management, such as the number of drains, location of placed drains, or drain type, varied widely according to institutional experience. There were cases which ultimately demonstrated no clinically relevant symptoms of pancreatic fistula despite having amylase-rich fluid on POD 1. In fact, 48.9% of patients with a drain amylase value >4,000 U/L on POD 1 did not develop a clinically relevant pancreatic fistula. Therefore, further studies are necessary to prospectively validate these predictive risk factors to confirm the possible relationship between these factors and the development of clinically relevant pancreatic fistula (grade B/C).

In conclusion, the results of this study, which was an initiative of the JSHPBS, indicated that male gender, soft pancreas, intraoperative bleeding >1,000 ml, and amylase value >4,000 U/L in drains on POD 1 were significant predictive risk factors for developing clinically relevant pancreatic fistula (grade B/C). Management of pancreatic fistula in the early period after PD is not sufficiently standardized. Therefore, the identification of these predictive risk factors can provide useful information to tailor the postoperative management for patients who are at an increased risk of developing pancreatic fistula, including drain management, and the administration of antibiotics, a protease inhibitor, octreotide, or enteral nutrition.

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Tumor-infiltrating CD4+ Th17 cells produce IL-17 in tumor microenvironment and promote tumor progression in human gastric cancer

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Abstract. Recently, a subset of IL-17 producing T cells distinct from Th1 or Th2 cells has been described as key players in inflammation and autoimmune diseases as well as cancer development. In this study, we investigated the expression level of IL-17 and T helper 17 (Th17)-related cytokines in gastric cancer tissues and assessed the association of their expression with angiogenesis and their clinicopathological parameters. Tumor and adjacent normal tissues were obtained from 82 patients with gastric cancer. IL-17, IL-21 and IL-23 mRNA expression levels were quantified by real-time RT-PCR. Th17 infiltration, microvessel density and neutrophil infiltration in tumor tissues were examined by immunohistochemistry and double immunofluorescence histochemistry. Expression of IL-17, IL-21 and IL-23 mRNA was found to be significantly up-regulated in tumor tissues compared with adjacent normal tissues. The expression level of IL-17 mRNA strongly and positively correlated with that of IL-21 mRNA in tumor tissue. The number of vascular endothelial cells and infiltrating neutrophils was significantly larger in tumors expressing a high level of IL-17 mRNA than in tumors expressing a low level of IL-17 mRNA. In tumor tissues most CD4+ cells were stained with anti-IL-17 antibody. The expression level of IL-17 mRNA in gastric tumors was associated with the depth of the tumors, lymph-vascular invasion and lymph node involvement, suggesting that IL-17 obviously was related to tumor progression. IL-17 and IL-21, which regulates IL-17, would be potential therapeutic targets for the treatment of gastric cancer.

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Key words: Th17, IL-17, IL-21, gastric cancer, angiogenesis, inflammation

Introduction

It has been established that cancer can be promoted or exacerbated by inflammation and infection. Chronic inflammation is a major driving force in tumor development (1-3). Interleukin (IL)-17 is considered a proinflammatory cytokine because it has been shown to increase the production of IL-6 and IL-8 in macrophages and fibroblasts (4-6). Recently a new lineage of effector CD4+ T cells characterized by production of IL-17, the T helper 17 (Th17) lineage, was described on the basis of developmental and functional features that are distinct from those of classic Th1 and Th2 lineages (7). The identification of this new subtype of Th17 cell has prompted renewed interest in IL-17 biology. IL-17 plays an important role in inflammation, and is critical in host defense against infectious disease, allergy, and autoimmune diseases such as rheumatoid arthritis and inflammatory bowel diseases, which include Crohn's disease and ulcerative colitis (8,9). Interestingly, IL-17 also has been reported to be up-regulated in *Helicobacter pylori* (Hp) infected gastric mucosa. IL-17 positively regulates the synthesis of IL-8 by gastric mononuclear cells and epithelial cells, which thus emphasizes the role of IL-17 in Hp-driven inflammation (10).

Recently, it has been reported that IL-17 promotes tumor growth through angiogenesis in mice (11). On the other hand, several reports have shown that IL-17 inhibits tumor growth through antitumor immunity in immunocompetent mice (12,13). It remains controversial whether IL-17 promotes or inhibits cancer progression. In humans, IL-17 expression has been reported in several tumor tissues such as ovarian cancer, colon cancer and also gastric cancer (14-16). Most solid tumors contain non-malignant cells, including immune cells and blood vessel cells, which are important in inflammation in the tumor microenvironment. In fact, a high percentage of CD4+ Th17 cells produce IL-17 at sites of ovarian cancer (17). However, in human tumors, the crucial molecular pathways that permit communication between abnormally growing cancer cells and these inflammatory cells remain unknown. In addition, the underlying mechanism of IL-17 at tumor sites in modulating tumor growth is still poorly understood.

Differentiation of Th17 cells from naïve T cells appears to involve signals from transforming growth factor β (TGF-β) and IL-6 (18,19). IL-21 has been reported to play an important role in the initial phase of Th17 differentiation (20,21). Although, in mice, there is a general agreement on the factors required for the generation of Th17 cells as mentioned above, the crucial initiating cytokines in humans for Th17 development remain unclear, and the relationship between IL-21 and IL-17 at tumor sites has not been elucidated.

In this study, we quantitatively investigated expression of IL-17 and IL-21 messenger RNA (mRNA) in gastric cancer tissues. In addition, we assessed the association of IL-17 expression levels with angiogenesis and neutrophil infiltration and its clinicopathological factors to clarify the role of tumor-infiltrating Th17 in tumor growth and progression. We also reviewed the possibility of IL-17 as a therapeutic target for patients with gastric cancer.

Materials and methods

Patients and tissue specimens. Included in the present study was a series of 82 patients (58 men, 24 women) with gastric cancer who underwent gastrectomy at Wakayama Medical University Hospital (WMUH) from 2004 to 2007. None of them received anticancer therapy before surgery. Individuals with concurrence of autoimmune disease, inflammatory bowel disease or viral infection were excluded. Clinicopathological characteristics of these 82 patients are summarized in Table I. Clinical stages of the tumors were determined according to the International Union Against Cancer TNM classification for gastric cancer. Samples of cancer tissues and non-cancerous adjacent tissues were collected from resected specimens of patients. Tumor samples were obtained from the invasive front of resected gastric cancer. Written informed consent was obtained from all patients before their participation in this study. In addition, the local ethics committee of WMUH approved this study.

RNA extraction and DNA synthesis. Total RNA was extracted with an RNeasy mini kit (Qiagen, Hilden, Germany) followed by RNase-Free DNase Set treatment (Qiagen). Complementary DNA was synthesized from 1 μg of total RNA by using the Reverse Transcription System (Promega) according to manufacturer's instructions.

Quantitative real-time RT-PCR. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed with isolated total RNA (1 μg) on the LightCycler system (Roche Molecular Biochemicals, Mannheim, Germany). The following oligonucleotide primers and hybridization probes were used: human IL-17 sense, 5'-CTGGGAAGACC TCATTGG-3' and antisense, 5'-CCTTTTGGGATTGGTA TTGG-3', fluorescein-labeled probe, 5'-TCCTCAGAATTT GGGCATCCTGGATTTC-3', and LC Red 640-labeled probe, 5'-TGGGATTGTGATTCCTGCCTTCACTATGG-3'; human IL-21 sense, 5'-AGGTCAAGATCGCCACAT-3' and antisense, 5'-TTTGCTGACTTTAGTTGGGC-3', fluorescein-labeled probe, 5'-CTGACCACTCACAGTTTGTCTCTACATCTTCTG GA-3', and LC Red 640-labeled probe, 5'-CTGGCAGAAA TTCAGGGACCAAGTCATTCA-3'; human IL-23 sense, 5'-GAGAAGCTGCTAGGATCG-3', and antisense, 5'-TGG TGACCCTCAGGCTGC-3', fluorescein-labeled probe, 5'-GCC TTCTCTGCTCCCTGATAGCCCTGTG-3', and LC Red 640-labeled probe, 5'-GCCAGCTTCATGCCTCCCTACTG GG-3'; human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense, 5'-TGAACGGGAAGCTCACTGG-3' and antisense, 5'-TCCACCACCCTGTTGCTGTA-3', fluorescein-labeled probe, 5'-TCAACAGCGACACCCACTCCT-3', and LC Red 640-labeled probe, 5'-CACCTTTGACGCTGGGGCT-3'. Primers and probes were designed by Nihon Gene Research

Table I. IL-17 mRNA expression and clinicopathological parameters.

Factor	No. of patients	Expression of IL-17 mRNA[a]	P-value
Gender			
Male	59	3.24±0.179	
Female	23	3.65±0.298	0.22
Age (years)			
<65	31	3.78±0.193	
≥65	51	3.11±0.212	0.118
Tumor stage[b]			
T0/T1	15	2.52±0.430	
T2	33	3.13±0.219	
T3	34	3.99±0.186	<0.005[a]
Histological type			
Differentiated	40	3.19±0.247	
Undifferentiated	42	3.52±0.188	0.658
Lymphatic invasion			
Negative	27	2.78±0.303	
Positive	55	3.65±0.164	<0.05[d]
Venous invasion			
Negative	43	2.95±0.226	
Positive	39	3.82±0.184	<0.05[d]
Lymph node metastasis			
Negative	40	2.95±0.234	
Positive	42	3.75±0.186	<0.05[d]
Stage[b]			
0/I	31	2.66±0.254	
II	21	3.63±0.297	
III	19	3.84±0.240	
IV	11	4.00±0.378	<0.05[c]
Tumor size (cm)			
<5	46	3.13±0.202	
≥5	36	3.63±0.232	0.068

[a]Expression of mRNA for IL-17 were corrected with GAPDH housekeeping control amplifications. Values represent mean ± SEM. [b]Stage according to the TNM classification for gastric cancer (UICC). [c]P-value of Kruskal-Wallis test as appropriate. [d]P-value of Mann-Whitney test as appropriate.

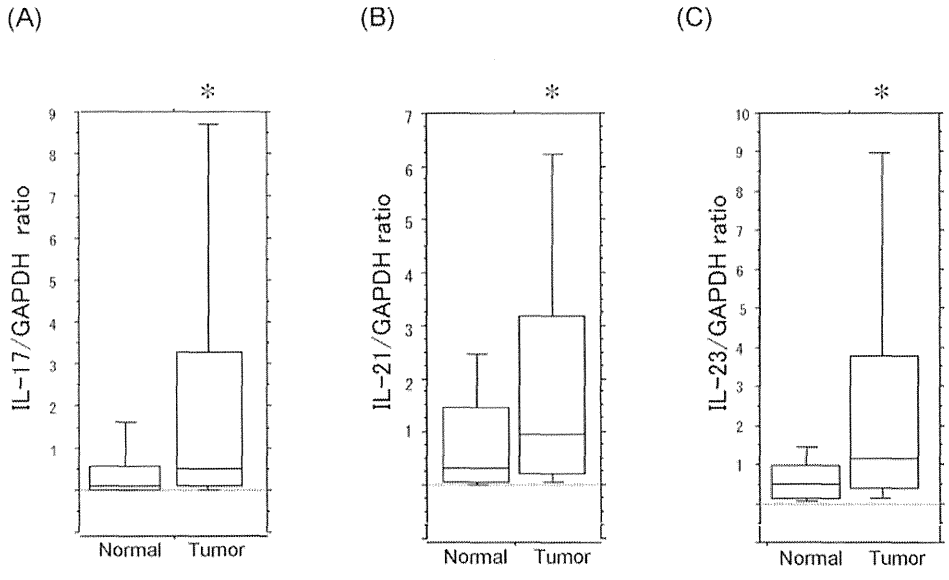

Figure 1. Level of IL-17, IL-21 and IL-23 mRNA expression in tumor and normal tissues of gastric cancer in 82 clinical samples. Expression levels of IL-17 (A), IL-21 (B) and IL-23 (C) mRNA were quantitatively determined by real-time RT-PCR for tumor tissue specimens and non-tumor tissue specimens from gastric cancer. Box plots show the 10th, 25th, 50th (median), 75th and 90th percentile values for log-transformed ratios of mRNA copies to GAPDH copies for IL-17, IL-21 and IL-23. *Significantly different from adjacent normal tissues ($p<0.0005$). Two-tailed p-values are based on the Mann-Whitney test.

Laboratories, Inc. (Miyagi, Japan). After 10 min of initial denaturation at 95°C, the cycling protocol entailed 40 cycles of denaturation at 95°C (10 sec), annealing at 62°C (15 sec) and elongation at 72°C (8 sec). For GAPDH, the thermocycling protocol was the same, except that annealing was performed at 55°C (15 sec) and 50 cycles were run. On each run, we quantified all samples according to the LightCycler software program, version 3.8 (Roche Molecular Biochemicals). The levels of mRNA for IL-17, IL-21 and IL-23 were corrected with GAPDH housekeeping control amplifications.

Immunohistochemistry and quantitative microscopy. Sections (4 μm) were prepared from paraffin-embedded blocks derived from gastric tumors. Sections were deparaffinized in xylene and graded alcohols, and rinsed in phosphate-buffered saline. Antigen retrieval from tissue was carried out by autoclaving the tissue in 0.01 M citrate buffer (pH 6.0) at 120°C for 10 min. The antibodies used included rabbit anti-IL-17 (dilution at 1:100, Santa Cruz Biotechnology); rabbit anti-IL-21 (dilution at 1:100, LifeSpan BioSciences); mouse anti-CD34, specific for endothelial tissue (dilution at 1:50, DakoCytomation, Glostrup, Denmark); and mouse anti-CD66b, specific for neutrophils (dilution at 1:500, BD Pharmingen). The antibodies were incubated overnight at 4°C. The immunocomplex was visualized by a polymer envision method, EnVision+ Kit (Dako). For quantification of tumor microvessel density and neutrophil infiltration, highly positive areas were initially identified by scanning tumor sections using light microscopy at low power. Areas of infarct-like necrosis and areas immediately adjacent to ulcerations were not considered in counts. Vessel counts were assessed according to the criteria of Weidner *et al* (22). Vessels in five high-power fields (x200 magnification) and neutrophil infiltration in five high-power fields (x400 magnification) were counted. Positive cells were quantified by an image processing application (Win ROOF, version 5.5; Mitani, Tokyo, Japan) and the manual counts were confirmed by a pathologist.

Double immunofluorescence histochemistry. Tissues were stained with primary antibodies: mouse anti-CD4 (dilution at 1:100, Dako) and rabbit anti-IL-17 (dilution at 1:100, Santa Cruz). The CD4 and IL-17 antibodies were detected with Alexa Fluor 488 conjugated goat anti-mouse immunoglobulin G (IgG) (Molecular Probes) and Alexa Fluor 546 conjugated goat anti-rabbit IgG (Molecular Probes). The double-stained sections were analyzed with a confocal laser scanning microscope (LSM5Pascal Exciter, version 4.0; Carl Zeiss, Jena, Germany).

Statistical analysis. The following statistical analyses were used. For data in Figs. 1, 3 and 4, we used the Mann-Whitney test. In Fig. 2, we used the Spearman rank correlation coefficient. In Table I, we used the Mann-Whitney test and the Kruskal-Wallis test. All statistical analyses were performed with StatView 6.0 (SAS Institute Inc) statistical software program. A value of $p<0.05$ was considered statistically significant.

Results

Expression of IL-17, IL-21 and IL-23 mRNA in tumor and non-cancerous tissues. IL-17 was found to be significantly up-regulated in tumor tissue compared with adjacent normal tissue ($p<0.0005$, Fig. 1A). Both IL-21 and IL-23, which are related to IL-17 production, were also significantly up-regulated in tumor tissue ($p<0.0005$, Fig. 1B and C).

Correlation of IL-17 mRNA with IL-21 and IL-23 mRNA in tumor tissues. The expression level of IL-17 mRNA positively correlated with that of IL-21 mRNA in tumor tissues ($r=0.730$,

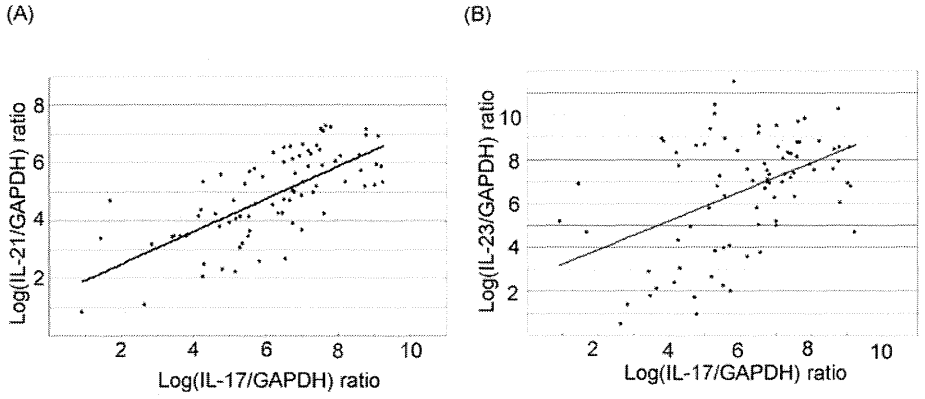

Figure 2. The correlation between expression levels of IL-17 mRNA, IL-21 mRNA and IL-23 mRNA. The correlation between expression levels of IL-17 mRNA and IL-21 mRNA [(A) r=0.730, p<0.0001], and the correlation between expression levels of IL-17 mRNA and IL-23 mRNA [(B) r=0.415, p<0.0005] were examined in tumor tissues. Log-transformed mRNA levels, normalized for GAPDH mRNA, are shown. The relationships are shown along with Spearman's rank-order correlation.

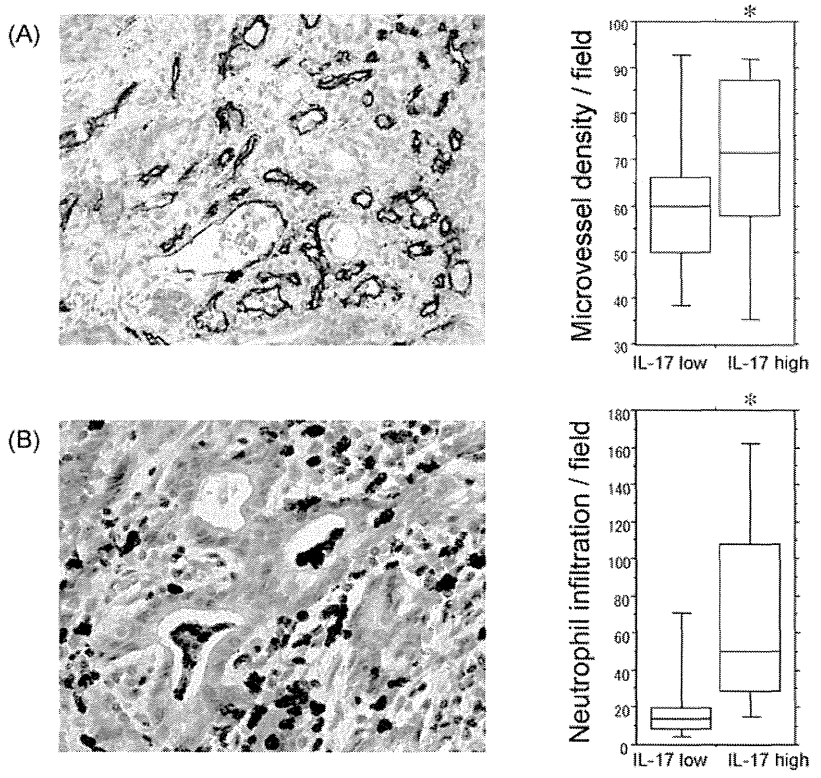

Figure 3. Microvessel density and neutrophils infiltration in tumor tissues. (A) Immunohistochemical staining of tumor tissues with anti-CD34 antibody, specific for endothelial tissues, was performed. Representative pictures of immunohistochemical staining of tumor tissues are shown. Vessels in five power fields (x200 magnification) were counted. (B) Immunohistochemical staining of tumor tissues with anti-CD66b antibody, specific for neutrophils, was performed. Representative pictures of immunohistochemical staining of tumor tissues are shown. Positive cells in five power fields (x400 magnification) were counted. Box plots show the 10th, 25th, 50th (median), 75th and 90th percentile values for the average number. *Significantly different from tumors expressing low levels of IL-17 (p<0.01). Two-tailed p-values are based on the Mann-Whitney test.

p<0.0001, Fig. 2A). On the other hand, there was no correlation between expression levels of IL-17 mRNA and IL-23 mRNA in tumor tissues (r=0.415, p<0.0005, Fig. 2B).

Quantification of tumor microvessel density. To assess the association between microvessel density and IL-17 expression in tumor tissue, we performed immunohistochemical staining with anti-CD34 antibody specific for endothelial cells. High- and low-expression groups were defined by the median value of IL-17 mRNA expression of this study population. The number of vascular endothelial cells was significantly higher in tumors expressing high IL-17 mRNA than in tumors expressing low IL-17 mRNA (p<0.01, Fig. 3A).

Quantification of tumor-infiltrating neutrophils. To assess the association between neutrophil infiltration and IL-17 expres-

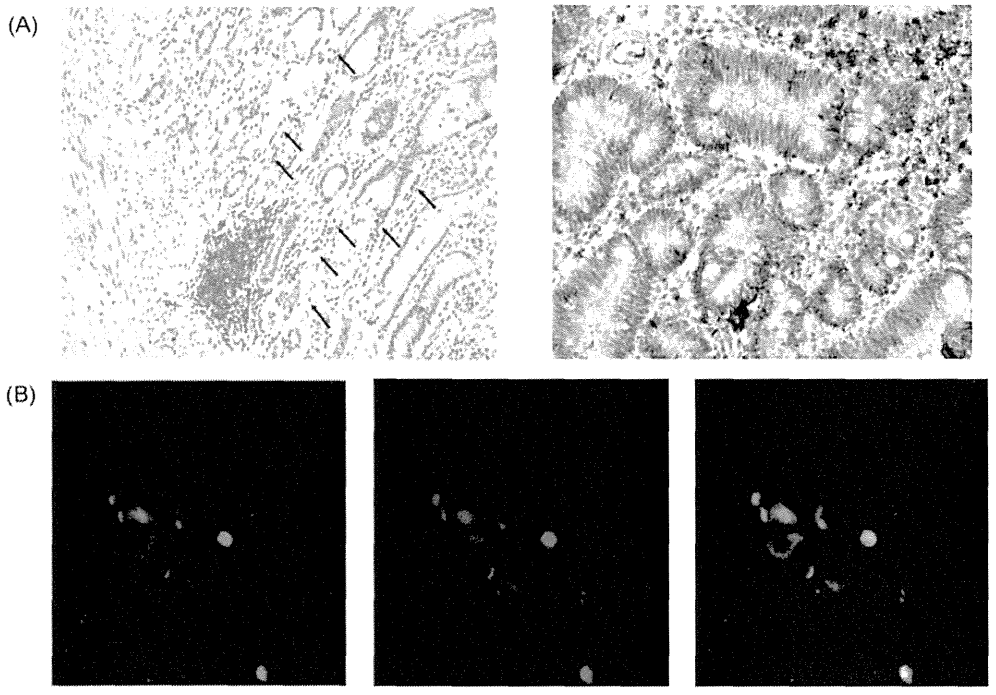

Figure 4. Immunohistochemistry for IL-17 in normal and tumor site. (A) Representative pictures of immunohistochemical staining with anti-IL-17 antibody at the normal (left panel) and tumor (right panel) site of gastric cancer specimens. More IL-17-positive cells were observed in the tumor tissues than in the adjacent normal tissues. The original magnification is x400. (B) Double immunofluorescence histochemistry for CD4 and IL-17 in tumor site. Tissue sections from a tumor were incubated with mouse monoclonal antibody against CD4 together with rabbit polyclonal antibodies against IL-17. The monoclonal antibody was detected with Alexa Fluor 488 conjugated goat anti-mouse IgG (green fluorescence; left panel), and the polyclonal antibodies were detected with Alexa Fluor 546 conjugated goat anti-rabbit IgG (red fluorescence; middle panel). Merged image of the two fluorophores is displayed in yellow (right panel). The original magnificationis x1000.

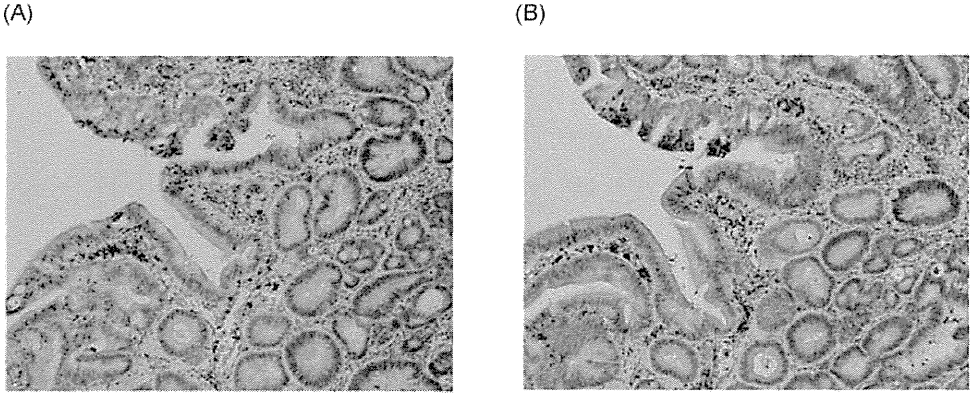

Figure 5. Immunohistochemistry for IL-17 and IL-21 in tumor site. (A) Representative pictures of immunohistochemical staining with anti-IL-17 antibody at the tumor site of gastric cancer specimens. (B) Representative pictures of immunohistochemical staining with anti-IL-21 antibody at the tumor site of gastric cancer specimens. The IL-21-positive cells did not always coincide with the IL-17-positive cells in the two serial sections. Original magnification x100.

sion in tumor tissue, we performed immunohistochemical staining with anti-CD66b antibody specific for neutrophils. High- and low-expression groups were defined by the median value of IL-17 mRNA expression of this study population. The number of infiltrating neutrophils was significantly lager in tumors expressing high IL-17 mRNA than in tumors expressing low IL-17 mRNA ($p<0.001$, Fig. 3B).

Distributions of IL-17-positive cells. To examine the expression of IL-17 in tumor and normal tissue, we performed immunohistochemistry for IL-17. IL-17 immunoreactive cells were rarely detected in non-cancerous adjacent tissues. On the other hand, there were abundant IL-17-expressing cells in tumor tissues; however, none of the tumor cells stained for IL-17 (Fig. 4A).

Identification of IL-17-producing cells in tumors. To identify IL-17-producing cells in tumor tissues, we performed double immunofluorescence histochemistry with anti-IL-17 and anti-CD4 antibodies in tumor tissues. The anti-CD4 primary

antibodies were detected with green fluorescence (Fig. 4B; left panel), and the anti-IL-17 primary antibodies were detected with red fluorescence (Fig. 4B; middle panel). A merged image of the two fluorophores, displayed in yellow, shows that most of the CD4+ cells in tumor tissues are stained with anti-IL-17 antibody (Fig. 4B; right panel). Therefore, Th17 cells that infiltrate tumor tissues produced IL-17.

IL-17 mRNA expression and clinicopathological characteristics. To evaluate the biological significance of IL-17 expression in tumor tissues in patients with gastric cancer, we investigated the association of mRNA expression levels of IL-17 with clinicopathological factors (Table I). IL-17 mRNA expression levels in gastric tumors were higher in positive groups of venous invasion, lymphatic invasion and lymph node metastases than in negative groups. IL-17 mRNA expression levels in gastric tumors increased according to the depth of invasion and the stage of disease. On the other hand, no significant association was recognized between the expression levels of IL-17 mRNA and histological type and tumor size.

Discussion

In this study, we quantitatively analyzed the expression of IL-17 mRNA in gastric tumor tissues. IL-17 was originally identified as a proinflammatory cytokine that induces neutrophils (4) and previous studies also have shown that inflammation is linked to cancer development and progression. Therefore, it is reasonable to speculate that proinflammatory Th17 cells may accumulate and produce IL-17 in the tumor microenvironment. Indeed, we showed in the present study that the expression level of IL-17 mRNA in tumor tissues was significantly higher compared with that in adjacent normal tissues. In addition, we examined IL-17 expression in tumor and normal tissues by immunohistochemistry, and showed that, in contrast to normal glands, malignant glands were surrounded by dense inflammatory cells including many IL-17-producing cells. This result was consistent with the data of IL-17 mRNA expression. Conventionally, it has been reported that IL-17 is predominantly produced by CD4+ helper T cells, which have been termed 'Th17' (5). However, recent studies have shown that T cells other than CD4+ T cells, such as γδT cells and CD8+ T cells, produce IL-17 in mouse models (23,24). In the present study, we performed double immunofluorescence histochemistry using anti-IL-17 and CD4 antibodies in tumor tissues to identify IL-17-producing cells in human gastric cancer tissues. We showed that most of the CD4+ cells infiltrating tumors were also stained with anti-IL-17 antibody, suggesting that IL-17 was predominantly produced by conventional Th17 cells in human gastric cancer tissues. Consistent with this observation, it has been reported that there is a high percentage of Th17 cells in tumor-derived T cell populations in 5 of 10 ovarian tumors, and those Th17 cells secreted IL-17 (17).

Differentiation of Th17 cells from naïve T cells is dependent on signals from TGF-β, IL-6, IL-21 and IL-23 in mice (21). Importantly, IL-21 is essential for the generation of Th17 cells and also amplifies Th17 cell differentiation (20). IL-23 does not act on naïve T cells, but instead acts on T cells that are already committed to the Th17 lineage. IL-23 enhances the production of IL-17 and stabilizes the Th17 phenotype (25,26). In humans, IL-21 likewise is required for differentiation of Th17 cells, and IL-23 enhances IL-17 secretion from those cells (27,28). However, there have been few studies to investigate the expression of IL-17-related cytokines such as IL-21 and IL-23, which play a crucial role in the generation of Th17 cells and the production of IL-17 in the tumor tissues of cancer patients. Our data showed that mRNA expression of both IL-21 and IL-23 was higher in gastric tumor tissues than in non-cancerous adjacent tissues. Importantly, in tumor tissues, the IL-21 mRNA expression level showed a strong correlation with the IL-17 mRNA expression level, but the IL-23 mRNA expression level did not show as strong a correlation with the IL-17 mRNA expression level. We also examined other cytokines such as TGF-β and IL-6, but we did not find a correlation as strong as that of IL-17 with IL-21 (data not shown). In addition, we performed immunohistochemical staining for IL-21 in tumor tissues and non-cancerous adjacent tissues to identify IL-21-producing cells. As with IL-17, there were many IL-21-positive cells around malignant glands, although the IL-21-positive cells did not always coincide with the IL-17-positive cells (Fig. 5). These results suggest that IL-21 may be crucial for the generation of Th17 cells and may play a pivotal role in the regulation of IL-17 secretion in the tumor microenvironment.

IL-17 is expressed in a considerable proportion of patients with ovarian cancer and its expression is associated with tumor angiogenesis (14). IL-17 up-regulates production of a variety of proangiogenic factors, such as vascular endothelial growth factor (VEGF), prostaglandin E1 (PGE1) and PGE2, and macrophage inflammatory protein-2 (MIP-2), by fibroblasts as well as tumor cells; IL-17 also promotes angiogenesis through stimulation of vascular endothelial cell migration and cord formation, resulting in tumor progression (11). The microvessel density and other angiogenic factors such as VEGF and PGE1, which are related to tumor development and progression, are predictive of patient survival in patients with gastric cancer. Therefore, it is important to examine the association of the expression level of IL-17 mRNA and angiogenesis in tumor tissues for the purpose of clarifying the biological significance of IL-17 in the tumor microenvironment. Our results revealed that tumor tissues with high expression levels of IL-17 mRNA had significantly higher microvessel density than those with low expression levels. Our findings strongly suggested that IL-17 released from tumor-infiltrating Th17 cells promoted angiogenesis by the production of proangiogenic chemokines in human gastric cancer. That process may lead to tumor progression.

From the standpoint of inflammation, IL-17 has the ability to stimulate IL-8 production in both epithelial cells and macrophages raising the possibility that this cytokine may play an important role in the recruitment of inflammatory cells in the tumor microenvironment. Neutrophils, acting alone or in concert with macrophages, also have been linked to tumor progression (2). Tumor-associated neutrophils promote tumor progression by a variety of mechanisms, including stimulation of angiogenesis and invasion (29). It has been shown in several tumor transplant models that tumor-associated neutrophils can stimulate tumor angiogenesis through production of

proangiogenic factors, including VEGF, IL-8 and certain proteases such as matrix metalloproteinases and elastases, and that these neutrophils can facilitate tumor progression. In this study, immunostaining for neutrophils revealed that tumor tissues with high expression levels of IL-17 mRNA had more infiltrating neutrophils than tissues with low expression levels. This observation suggested that IL-17 from Th17 recruited neutrophils into the tumor microenvironment, and that these tumor-associated neutrophils may contribute to the invasive potential.

There are conflicting data on a possible role in carcinogenesis in mouse models. Forced overexpression of IL-17 ectopically in tumor cells can either suppress tumor progression through enhanced antitumor immunity (12,30) or promote it through an increase in inflammation and angiogenesis (11). The role of endogenous IL-17 in tumorigenesis has been assessed with IL-17 knockout mice (13). Although the results are still controversial, they give useful information for clarifying whether IL-17 promotes or inhibits tumor progression in human cancer and for investigating the association of locally expressed IL-17 and clinicopathological characteristics in patients with gastric cancer. In this study, we focused on the clinical significance of IL-17 in the tumor microenvironment. To our knowledge, no prior reports exist on the correlations between locally expressed IL-17 and clinicopathological factors of cancer patients from this point of view. Although Zhang *et al* reported that IL-17 was significantly increased in serum and tumor-draining lymph nodes in patients with advanced gastric cancer (16), locally expressed IL-17 would have quite a different role in tumor progression. This study is the first to report the clinical significance of locally expressed IL-17 in gastric cancer. Clinicopathological analysis revealed that patients with high IL-17 mRNA expression level showed a deeper invasion of tumors into the wall, more lymph-vascular invasion and more positive lymph node involvement than those with low expression. IL-17 at the tumor sites may thus play a vital role in proliferation and progression of gastric cancer.

In conclusion, our findings showed that $CD4^+$ Th17 cells were generated around the tumor, and that Th17 cells infiltrated the tumor and secreted IL-17, leading to tumor progression through angiogenesis and neutrophil infiltration in patients with gastric cancer. One of the key cytokines to regulate the production of IL-17 in the tumor microenvironment was thought to be IL-21. It was suggested that IL-17 or IL-21 locally expressed in the tumor would be a potential therapeutic target for treatment of patients with advanced gastric cancer.

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Comparison of different classes of CpG-ODN in augmenting the generation of human epitope peptide-specific CTLs

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Abstract. Three distinct classes of CpG-oligonucleotides (ODN) (CpG-A, CpG-B and CpG-C) have been identified on the basis of differences in their structures and immune effects. To date, only CpG-B is applied for clinical treatments; however, it is still unknown which of the different CpG-ODN classes is most useful as an adjuvant for human cancer vaccine therapy. In the present study, we examined the activity of these 3 types of CpG-ODN in enhancing the induction of human peptide-specific CTLs. Our data showed that the specific cytotoxicity was augmented in the presence of CpG-A, -B and -C but not in the presence of control ODN, and the augmenting effect was most potent with CpG-A. Flow cytometric analysis showed the subpopulation of effector-memory cells in $CD8^+$ cells was most increased with CpG-A. Furthermore, depletion of PDCs from PBMCs before stimulation with peptide and CpG-ODN completely abrogated the augmenting effect of CpG-ODN. These data indicated that the stimulation of PDCs by CpG-ODN augmented the generation of peptide-specific CTLs, and CpG-A was superior to CpG-B and CpG-C in terms of augmenting the generation of human peptide-specific CTLs *in vitro*.

Introduction

The innate immune system is activated via exposure to pathogen-associated molecular patterns (PAMPs) that are expressed by a diverse group of infectious microorganisms. Subsequently, the host mounts an adaptive immune response directed against determinants that are uniquely expressed by the pathogen. The resultant antigen-specific immunity is characterized by the production of high-affinity antibodies and the generation of cytotoxic T cells that provide long-lasting protection (1). The key feature of innate immune cells that enables them to detect and categorize infection seems to be their repertoire of what have been termed pattern-recognition receptors (PRRs). The best understood family of PRRs is the toll-like receptors (TLRs), of which 10 are known in humans.

In contrast to viruses and other pathogens, vaccines containing recombinant proteins or synthetic antigenic peptides usually fail to induce significant immune responses unless they are mixed with adjuvant (3,4). Because of their high efficacy, several recently identified TLR ligands are promising vaccine adjuvants. Bacterial unmethylated CpG-rich oligodeoxy-nucleotides (ODN), which bind to TLR-9, are one of the most promising candidates for a cancer vaccine adjuvant and are currently being tested in many human clinical trials (5-8).

In numerous murine models, TLR-9 activation enhances antigen-specific cellular responses to a wide variety of antigens. The mechanism that contributes to the potent adjuvant activity of CpG-ODNs is maturation and differentiation of dendritic cells resulting in the strong induction of CTLs, even in the absence of CD4 T-cell help (9). On the other hand, the cellular patterns of TLR expression vary between different species (2,10). B cells, monocytes and all DC subsets express TLR-9 in mice; however, only plasmacytoid dendritic cells (PDC) and B cells express TLR-9 in humans (11-14). Consequently, the murine immune system produces different actions from human systems when exposed to CpG-ODN. Therefore, it is impossible to extrapolate the experimental results from murine models to humans. Furthermore, little is known about the mechanism by which CpG-ODNs augment acquired cellular immunity in humans, although systemically administered CpG-ODNs have shown substantial evidence of augmenting the activity of anti-tumor immunity in human clinical cancer vaccine trials (5-8).

Three distinct classes of CpG-ODN have been identified on the basis of differences in their structures and immune-stimulating effects (9,15-17). CpG-A induces the production of high levels of IFN-α from PDC with relatively little B-cell stimulation. In contrast, CpG-B induces the production of low levels of IFN-α along with profound B-cell activation. CpG-C has intermediate immune effects with excellent *in vivo* stability and ease of formation. To date, only CpG-B has been applied for clinical treatments; however, the class of CpG-ODN

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that is most useful as an adjuvant for a human cancer vaccine is still unknown.

In the present study, we examined the immuno-modulatory activity of these 3 types of CpG ODN in terms of the generation of peptide-specific CTLs.

Materials and methods

Cell lines. A24+LCL cells (HLA-A24/24) were a generous gift of Takara Shuzo Co., Ltd. (Otsu, Japan). The A24LCL cells were used for peptide-mediated cytotoxicity assays. These cells were maintained in a tissue culture flask using RPMI and supplemented with antibiotics and 10% heat-inactivated fetal calf serum (Gibco BRL).

Oligodeoxynucleotides. CpG-A was synthesized by Gene Design (Osaka, Japan). CpG-B, CpG-C and GpC-ODN were synthesized by Hokkaido System Science (Sapporo, Japan); CpG-A, 5'-ggTGCAT**CG**ATGCAGGGGgg-3'; CpG-B, 5'-**tcgtc gttttgtcg**ttttgtcgtt-3'; CpG-C, 5'-**tcgtcg**aa**cg**ttcgagagatgat-3'; GpC-ODN, the GC control to CpG-ODN, 5'-ggTGCATG CATGCAGGGGgg-3' (lower case letters indicate phosphorothioate linkage; capital letters, phosphodiester linkage 3' of the base; bold, CpG-dinucleotides).

Peptides. Peptide derived from the squamous cell carcinoma-associated differentiation antigen LY6K-177 (RYCNLEGPPI), influenza (flu) virus-derived peptide (RFYIQMCYEL) and HIV-derived peptide (RYLRDQQLL) with the HLA-A24 binding motif were purchased from Takara Bio Inc. (Otsu, Japan). The purity (>90%) and the identity of the peptides were determined by analytical HPLC and mass spectrometry analysis, respectively. Peptides were dissolved in dimethylsulfoxide (DMSO) at 20 mg/ml and stored at -80°C.

Cytokine assays. Human PBMCs from healthy volunteers (n=15) were isolated from freshly drawn peripheral blood by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation. Blood donors were negative for HIV, hepatitis B virus (HBV) and HCV infection.

Freshly isolated PBMC ($1x10^6$ in 500 μl of AIM-V media, Invitrogen) were incubated at 37°C with 5% CO_2 in 48-well flat-bottom plates with each class of CpG-ODN at different concentrations. Cell supernatants collected after 48 h were stored at -80°C until assayed. IFN-α in cell supernatants was measured by ELISA according to the manufacturer's instructions (PBL Biomedical Laboratories, Piscataway, USA). All assays were performed in triplicate.

Induction of flu peptide-specific CTLs. For this study, HLA-A24-positive donors were selected. PBMCs ($2x10^6$/ml) isolated from healthy volunteers were stimulated with the flu peptide at a concentration of 10 μg/ml in the presence of CpG-ODNs (20, 5 and 5 μg/ml for CpG-A, -B and -C, respectively) in 24-well culture plates in AIM-V containing 2% heat-inactivated autologous serum (AS). In some experiments, PBMCs were depleted of PDCs using BDCA4-coupled magnetic beads (Miltenyi Biotec) according to the manufacturer's protocol (<0.01% PDCs identified as $BDCA2^+$ and $CD123^+$ after depletion) to investigate the role of PDCs on the adjuvant effect of CpG-ODNs. On day 7, the T cells were further stimulated with the peptide-pulsed adherent cells that were cultured with autologous irradiated PBMCs for 4 h. The cytotoxic activity of CTLs was tested against peptide-pulsed A24-LCL cells on day 14 as indicated.

Induction of LY6K peptide-specific CTLs. Monocyte-derived dendritic cells (DCs) were used as antigen-presenting cells to induce CTLs against peptides presented on HLA. DCs were generated *in vitro* as previously described (18). Briefly, PBMCs isolated from healthy volunteers (HLA-A*2402) were separated by adherence to a plastic tissue culture dish (Becton-Dickinson) so as to enrich them for the monocyte fraction. The monocyte-enriched population was cultured in the presence of 1,000 U/ml GM-CSF (Kirin) and 500 U/ml IL-4 (Ono) in AIM-V containing 2% heat-inactivated AS. After 5 days in the culture, TNF-α (10 ng/ml), IL-6 (1,000 U/ml), IL-1β (10 ng/ml) and PGE2 (1 μg/ml) were added to the culture to mature DCs. After 7 days, DCs were pulsed with 20 μg/ml of the synthesized peptides in the presence of 3 μg/ml β2-microglobulin (Sigma), pulsed on the cytokine-generated DCs for 4 h at 37°C in AIM-V. These peptide-pulsed DCs were then inactivated by γ irradiation (50 Gy) and used as stimulator cells. To increase the precursor frequency of peptide-specific cells, $CD8^+$ T cells were enriched by one round of positive selection using anti-CD8 antibody beads and MACS technology according to the manufacturer's protocol (Miltenyi Biotec; Bergisch-Gladbach, Germany). Then, $CD8^+$ T cells and unseparated PBMCs were mixed at a 1:2 ratio and used as the responder cells. These cultures were set up in 48-well plates (Corning); each well contained $5x10^4$ stimulator cells and $1.5x10^6$ responder cells in the presence of CpG-ODNs in 0.5 ml of AIM-V/2% AS. In some experiments, PBMCs were depleted of PDCs using BDCA4-coupled magnetic beads (Miltenyi Biotec) according to the manufacturer's protocol. Two days later, these cultures were supplemented with IL-2 to a final concentration of 20 IU/ml. On day 7, the T cells were further re-stimulated with the autologous peptide-pulsed DCs. The peptide-pulsed DCs were prepared in the same manner as described above. CTL activity was tested against peptide-pulsed A24-LCL cells on day 14 as indicated.

Cytotoxicity assay. Target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Perkin-Elmer Life Sciences) for 1 h at 37°C in a CO_2 incubator. Peptide-pulsed targets were prepared by incubating the cells with 20 μg/ml of the peptide for 16 h at 37°C before labeling. Labeled target cells were rinsed and mixed with effector cells in a final volume of 0.2 ml in round-bottom microtiter plates. The plates were centrifuged (4 min at 800 x g) to increase cell-to-cell contact and placed in a CO_2 incubator at 37°C. After 4 h of incubation, 0.1 ml of the supernatant was collected from each well and the radioactivity was determined with a γ counter. The percentage of specific cytotoxicity was determined by calculating the percentage of ^{51}Cr release in 4 h using the following formula: [(cpm of the test sample release - cpm of the spontaneous release)/(cpm of the maximum release - cpm of the spontaneous release)] x 100. Spontaneous release was determined by incubating the target cells alone, in the absence of effector cells, and the maximum release was obtained by incubating the targets with 1 N HCl. All measurements were carried out in triplicate and the standard

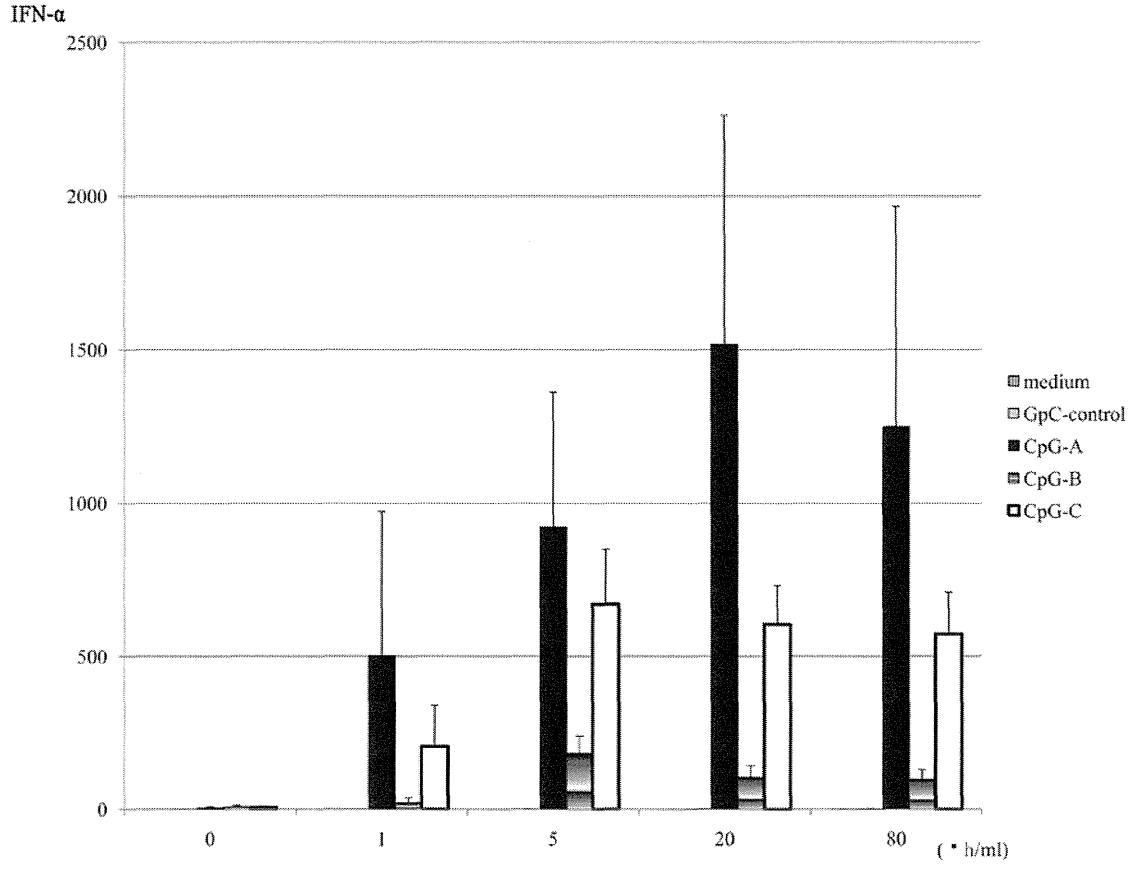

Figure 1. IFN-α secretion from PBMCs stimulated by CpG-ODNs. Levels of IFN-α secreted by PBMC from healthy donors (n=15) after 48-h culture with media, GpC control ODN, A-Class, B-Class or C-Class CpG (all ODN at 0, 1, 5, 20, 80 μg/ml). Bars show mean values and standard error of the means for each group of subjects.

errors of the means were consistently below 10% of the value of the mean.

Flow cytometric immunofluorescence analysis. Monoclonal antibodies against human CD8PerCP, CD45RAFITC and CCR7PE were purchased from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA). Cells were incubated with specific antibodies in PBS for 30 min at 4°C, then analyzed using a FACSCalibur with the Cell Quest software package (Becton-Dickinson).

Results

IFN-α secretion from PBMCs stimulated by CpG-ODNs. Distinct ODN classes were studied for their ability to stimulate human PBMCs to secrete IFN-α. Consistent with previous reports (15-17), IFN-α secretion from PBMCs was greatest with CpG-A, and it was moderate with CpG-C; it was lowest with CpG-B. The dosages of CPG-A, B and C that induced the maximum level of IFN-α were 20, 5 and 5 μg/ml, respectively (Fig. 1).

The effect of CpG-ODNs on the induction of the influenza peptide-specific CTL. The flu peptide-specific CTLs showed clear cytotoxicity against flu peptide-pulsed A24+LCL but not against HIV peptide-pulsed A24+LCL (Fig. 2A and B). The cytotoxicity against flu peptide-pulsed A24+LCL was augmented in the presence of CpG-A, -B and -C but not in the presence of control ODN, and the augmenting effect was greatest with CpG-A; it was moderate with CpG-C and low with CpG-B (Fig. 2A). The depletion of PDCs from PBMCs before stimulation with peptide and CpG-ODNs completely abrogated the augmenting effect of each class of CpG-ODN (Fig. 2C-E). These data indicated that the stimulation of PDCs by CpG-ODNs augmented the expansion and activation of flu peptide-specific CTL to increase the specific cytotoxicity.

Flow cytometric analysis showed the population of $CD8^+$ cells in flu peptide-specific CTLs. Interestingly, the subpopulation of effector-memory cells in $CD8^+$ cells was most increased with CpG-A, and moderately increased with CpG-C (Fig. 3).

CpG-A augmented the LY6K peptide-specific CTL induction in a PDC-dependent manner. The population of LY6K peptide-specific CTL precursor cells in healthy volunteers may be much smaller than that of flu peptide-specific CTLs because LY6K is a cancer-testis antigen (19). Therefore, we investigated whether CpG-ODNs could also affect the induction of LY6K-specific CTLs. Although the induction of influenza-specific CTL was augmented by all types of CpG-ODN (Fig. 2A), the LY6K peptide-specific CTLs were induced only with CpG-A from both donors 1 and 2, but not with CpG-B or CpG-C (Fig. 4A-D). Depletion of PDCs from PBMCs of donor 2 before stimulation with peptide and CpG-A completely abrogated the effect of CpG-A (Fig. 4E).