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CASE REPORT

Gastrointestinal bleeding during anti-angiogenic peptide vaccination in combination with gemcitabine for advanced pancreatic cancer

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Abstract Most pancreatic cancer patients are diagnosed at the advanced stages, and no therapy is superior to gemcitabine alone. To confirm the feasibility and efficacy of a novel clinical intervention using tumor vessel-specific anti-angiogenic peptide vaccination, we conducted a clinical phase I/II trial using HLA-A*2402/A*0201-restricted vascular endothelial growth factor receptor type 1 (VEGFR1)-derived peptide vaccination in combination with gemcitabine for advanced pancreatic cancer (http://www.clinical-trials.gov; NCT00683358 and NCT00683085). Four of the enrolled patients ($n = 2$ for HLA-A*2402 and $n = 2$ for HLA-A*0201 protocol, respectively), defined as having progressive disease according to the Response Evaluation Criteria in Solid Tumors version 1.0 (RECIST v.1.0), failed to respond to the therapy. Another two patients enrolled in HLA-A*2402 protocol dropped out of the study due to rapid disease progression. Grade 2–3 hematologic toxicities were observed in all cases, but the treatment was well tolerated with minimal systemic adverse events. One case in HLA-A*2402 protocol and another case in HLA-A*0201 protocol suffered complicated gastrointestinal (GI) bleeding during vaccination. The causal relationship between GI bleeding and VEGFR1-peptide vaccination is unclear according to the pathologic examination. These studies terminated prematurely because of the advanced stage of the disease in the enrolled patients on entry to the study. Despite GI bleeding, peptide vaccination provides a feasible treatment option for many advanced pancreatic cancer patients.

Keywords Pancreatic cancer · Peptide vaccination · VEGFR1 · Gemcitabine · GI bleeding

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Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States, and over 80% of cases are diagnosed in an advanced stage [1]. In spite of intensive research and the development of novel anticancer drugs, mortality from pancreatic cancer has not improved for decades [2]. Therapeutic intervention is very limited even in the gemcitabine (GEM) era, although GEM is the gold standard therapy against advanced pancreatic cancer [3]. Numerous novel anticancer drugs, including capecitabine, cisplatin, oxaliplatin, topoisomerase inhibitors (CPT-11, exatecan), mammalian target of rapamycin (mTOR)

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inhibitors, farnesyltransferase inhibitors, thymidylate synthase inhibitors, and matrix metalloproteinase inhibitors, with or without GEM, failed to prove their efficacy over that of GEM alone [4]. Erlotinib, an oral human epidermal growth factor receptor type 1 (HER1/EGFR) tyrosine kinase inhibitor, attained the minimal elongation of overall survival (OS) in combination with GEM, compared to GEM alone (median OS, erlotinib with GEM 6.24 months vs. GEM alone 5.91 months; $p = 0.038$) [5], while axitinib did not show such a survival effect [6, 7]. At present, no combination chemotherapy is available that is superior to GEM alone.

Since the first description by Folkman [8], anticancer strategy specifically targeting tumor angiogenesis has come of age [9]. Vascularization is completed during the embryonic developmental stage of ontogeny, so that adult angiogenesis occurs only in limited circumstances such as local ischemic conditions, inflammation, and tumor angiogenesis. The strategy of selectively targeting tumor vessels allows highly specific and effective cancer treatment with minimal toxicity. Vascular endothelial growth factor (VEGF), initially cloned as a heparin-binding growth factor, is specifically expressed on vascular endothelia [10], and identified as a homolog of vascular permeability factor (VPL) [11]. VEGF plays a crucial role in neovascularization and angiogenesis of tumor vessels [9]. One of its receptors, VEGF type 1 receptor (VEGFR1), is a member of the membrane-bound cytokine receptor superfamily [12]. VEGFR1 is specifically expressed on tumor vessels and contributes to tumor angiogenesis [9]. It is also considered to be involved in pancreatic cancer cell progression and migration [13, 14].

The anti-VEGF monoclonal antibody bevacizumab [15–34] and the multi-targeted tyrosine kinase inhibitors sorafenib [35, 36] and sunitinib [17, 37] produce an effective tumor response for various types of cancers. Their toxicities are relatively limited, but epistaxis is common after bevacizumab administration [15–34]. Severe bleeding events have also been described [16–38]. Ishizaki et al. [39] showed that the human VEGFR1-derived HLA-A24-restricted peptide (SYGVLLWEI) induces cytotoxic T-lymphocyte (CTL) responses in vitro using peripheral blood mononuclear cells (PBMNCs) from healthy volunteers. They established healthy donor-derived CTL lines by stimulating PBMNCs with this peptide, and these CTL lines specifically killed the target cells in vitro in a HLA-class-I-restricted manner. They reported that the HLA-A*0201-restricted VEGFR1-derived peptide VEGFR1-A2-770 (TLFWLLLTL) also induces specific immune responses in the PBMNCs of healthy volunteers in vitro and in HLA-A2-transgenic mice in vivo. VEGFR1-A2-770 peptide vaccination induced tumor regression in a HLA-A2 transgenic murine model. Among the Japanese population, 60.8% and 19.9% share a common HLA-A*2402 allele and HLA-A*0201 allele, respectively [40]. Thus, we conducted a HLA-A*2402- or HLA-A*0201-restricted VEGFR1-derived peptide vaccination trial in HLA-A*2402- or HLA-A*0201-positive Japanese pancreatic cancer patients who were resistant to conventional chemotherapy and had no alternative effective treatment.

Case report

Object of the study

To evaluate the feasibility and efficacy of combined modality intervention using GEM with HLA-A*2402-restricted VEGFR1-restricted peptide (VEGFR1-A24-1084; SYGVLLWEI) or HLA-A*0201-restricted VEGFR1-restricted peptide (VEGFR1-A2-770; TLFWLLLTL) vaccination, we conducted a phase I/II clinical trial for advanced chemotherapy-resistant pancreatic cancer with no alternative therapy. The primary outcome measure for phase I was safety assessed according to the National Cancer Institute Common Terminology Criteria of Adverse Event version 3 (NCI-CTCAE v.3), and that for phase II was time to progression (TTP) according to the Response Evaluation Criteria in Solid Tumors version 1.0 (RECIST v.1.0). Secondary outcome measures were immune response and tumor regression. This protocol was reviewed and approved by the institutional review board (IRB; approval number 20-24 for HLA-A*2402 and approval number 20-23 for HLA-A*0201) and submitted to ClinicalTrials.gov (http://www.clinicaltrials.gov; NCT00683358 and NCT00683085). Written informed consents were obtained from all eligible patients according to the Declaration of Helsinki.

Patients

Eligibility criteria were as follows: (1) advanced pancreatic cancer patients from 20 to 85 years of age with any prior therapy and no alternative effectiveness-proven therapy (locally advanced or metastatic inoperable cases and recurrent cases after surgery or chemotherapy were included); (2) patients who were heterozygous or homozygous for HLA-A*2402 allele for HLA-A*2402-restricted peptide or HLA-A*0201 allele for HLA-A*0201-restricted peptide, determined by PCR-based mid-high resolution HLA-DNA typing; (3) patients with an Eastern Cooperative Oncology Group (ECOG) performance status of 0–2; (4) patients with evaluable primary or metastatic lesions by RECIST v.1.0 criteria; (5) patients who could have a washout period of at least 4 weeks for prior anticancer therapy before study entry; (6) patients with life expectancy of >3 months; and (7) patients with adequate bone

marrow, hepatic, renal functions as follows: white blood cell count >2,000/μl, platelet count >100,000/μl, aspartyl aminotransferase (AST) <150 IU/L (institutional normal value is ≤40 IU/L), alanyl aminotransferase (ALT) <150 IU/L (institutional normal value is ≤44 IU/L), total bilirubin <3.0 mg/dl, serum creatinine <3.0 mg/dl. Patients with active uncontrolled infection, systemic use of corticosteroids or other immunosuppressants, uncontrolled brain metastasis or disseminated meningeal metastasis, unhealed external wound, paralytic ileus or interstitial pneumonitis, as well as pregnant or breastfeeding patients, were excluded.

Vaccination protocol

GEM 1,000 mg/m^2 body surface area was intravenously administered on days 1, 8, 15, 29, 36, and 43, either in our hospital or at the outpatient clinic. HLA-A*2402-restricted VEGFR1-derived peptide (VEGFR1-A24-1084; SYGVLLWEI) was purchased from AmbioPharm, Inc. (North Augusta, SC, USA). HLA-A*0201-restricted (VEGFR1-A2-770; TLFWLLLTL) peptide was purchased from NeoMPS, Inc. (currently a part of PolyPeptide Laboratories, San Diego, CA, USA). We emulsified 1 mg of these peptides with 1 ml of incomplete Freund's adjuvant (IFA; Montanide *ISA51, SEPPIC S.A., Puteaux, France). Vaccines were administered twice weekly by subcutaneous injection into 1 of 6 sites including bilateral cervical, axillary, and inguinal nodal areas in rotation.

Imaging study

Target lesions, non-target lesions, and new lesions were evaluated before and after 16 doses of peptide vaccine by contrast-enhanced computed tomography (CT) and diagnosed on the basis of RECIST v.1.0. One case (ID 24021) of rapid disease progression underwent CT before completion of the vaccination regimen and was diagnosed as progressive disease (PD) on day 29.

Immunological assays

One day before vaccination, and on day 29 and day 56, patients' PBMNCs were collected and cryopreserved. These cells were used for flow cytometric analysis and enzyme-linked immunosorbent spot (ELISpot) assay after 2 cycles of CD4 depletion using Dynabeads CD4^{TM} (Veritas Inc, Tokyo, Japan) according to the manufacturer's instructions. T2 cell line (American Tissue Culture Collection, Manassas, VA, USA) for HLA-A*0201-positive patients' PBMNC and TISI cell line (Takara Bio Inc., Shiga, Japan) for HLA-A*2402-positive patients' PBMNCs were used as stimulator cells. To detect the HLA-A*0201- or HLA-A*2402-specific T cell receptor (TCR) positive lymphocytes, thawed PBMNCs were CD4-depleted and stimulated in vitro with HLA-A*0201-VEGFR1-peptide with T2 cell line for HLA-A*0201 patients' PBMNCs or HLA-A*2402-VEGFR1-peptide with TISI cell line for HLA-A*2402 patients' PBMNCs for three days. After in vitro stimulation, HLA-A*0201-VEGFR1-peptide (TLFWLLLTL)-dextramer complex with RPE probe and HLA-A*2402-VEGFR1-peptide (SYGVLLWEI)-dextramer complex with RPE probe (Immudex, Copenhagen, Denmark) were used. As a negative control dextramer, HLA-A*0201-restricted HIV-specific (ILKEPVHGV) dextramer−RPE and HLA-A*2402-restricted HIV-specific (RYLRDQQLL) dextramer−RPE were used for analysis. FITC-conjugated anti-human CD8 mouse monoclonal antibody (clone RPA-T8), APC-conjugated anti-human CD3 mouse monoclonal antibody (clone; UCHT1), and FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA) were used for analysis. The ELISpot PLUS kit for human interferon-γ (MABTECH AB, Nacka Strand, Sweden) was used according to the manufacturer's instructions to determine peptide-specific interferon-γ release. Responder (patients' PBMNCs) and stimulator (TISI cell line for HLA-A*2402 and T2 for HLA-A*0201) ratios (R/S ratio) were serially diluted as 1:1, 1:2, 1:4, 1:8, respectively. Positive spots at R/S ratio 1:1, with or without peptide, are shown in Table 2. All experiments were triplicated and the average number of spots with standard deviation was indicated.

Case 1

We enrolled 4 eligible patients into a HLA-A*2402 trial (NCT00683358, Table 1). Two patients (ID 24021 and 24022) died before completion of 16 doses of vaccine due to rapid disease progression, and one patient (ID 24024) dropped out of the study due to cancer-induced intestinal obstruction. Therefore, only Case 1 (ID 24023) completed 16 doses of peptide vaccine. The patient was a 69-year-old female with a previous history of uterine cancer (clinical stage Ib, mixed cancer of endometrioid type and serous adenocarcinoma of the uterus body) complicated with ovarian cancer (clinical stage Ia, mucinous adenocarcinoma of the left ovary) 4 years before the diagnosis of pancreatic cancer. During regular outpatient follow-up of her uterine cancer, an elevation of CA19-9 was observed, and further evaluation revealed that she had a pancreatic body tumor. She underwent a pancreaticoduodenectomy in January 2007. Intraoperative staging was T2N0M0, and a diagnosis of invasive ductal carcinoma of the pancreas body was confirmed based on pathologic findings. After surgery, she received 16 doses of adjuvant GEM 1,000 mg/m^2

Table 1 Brief summary of enrolled cases

	A*2402 protocol				A*0201 protocol	
Patient ID number	24021	24022	24023	24024	02011	02012
Age at entry	55	50	69	66	50	60
Gender	M	M	F	M	M	M
Stage at diagnosis	IV	IV	IV	IV	IV	IV
Pathologic confirmation	−	−	+	−	−	−
Metastatic sites	Liver, LNs	Liver, LNs	P, LNs	M, P	Liver	Liver, lung
Sum of target lesions (mm) before vaccination	295.2	148.9	39.3	15.1	84	206.7
Sum of target lesions (mm) after vaccination	353.6[a]	NE	44.2	NE	118.8	287.8
New lesions	Yes	NE	Yes	NE	Yes	Yes
Total no. of vaccinations	9	2	16	6	18	16
Completion of vaccination therapy	No	No	Yes	No	Yes	Yes
Clinical response	PD	NE	PD	NE	PD	PD
TTP (days)	29	4	59	20	55	68

LNs lymph node metastasis; *M* mesenteric nodules; *P* peritoneal dissemination; *NE* not examined; *PD* progressive disease; *TTP* time to progression; the sum of tumor diameters was evaluated by contrast-enhanced CT based on the Response Evaluation Criteria in Solid Tumors (RECIST 1.0) before and after 16 doses of vaccine

[a] Patient ID 24021, the sum of tumor diameters after vaccination was evaluated on day 29 by CT, because of marked progression of the disease and patient debilitation

biweekly for 8 months. A contrast-enhanced CT scan indicated enlargement of disseminated peritoneal metastatic nodules. Her attending doctor considered that she had failed to respond to GEM, and decided to switch the chemotherapy to S-1 oral administration of 80 mg/day for 2 weeks, followed by 2 weeks rest, but mesenteric dissemination progressed after 4 months of S-1 therapy. An intravenous injection of 6 mg mitomycin C every 3 weeks was added to S-1 from February 2008, but the disease progression and the CA19-9 elevation continued. She first visited our hospital in June 2008, for screening for eligibility to our protocol. A CT scan revealed a pelvic mass compressing her left ureter, causing left hydronephrosis. After catheterization of the intact right ureter to avoid bilateral obstruction and to preserve residual renal function, she received 6 doses of GEM in combination with 16 doses of HLA-A*2402-restricted VEGFR1-derived peptide vaccine from August 2008.

After the first peptide vaccination, the patient complained of hematochezia. Colonoscopy detected the direct invasion of pelvic pancreatic cancer to the rectosigmoid colon (Fig. 1). We intravenously administered 6,000 U of erythropoietin weekly for hemorrhagic anemia. During the 16-dose vaccination regimen, grade 1 thrombocytopenia, grade 2 leukopenia, grade 2 neutropenia, grade 3 lymphopenia, and grade 1 injection site reaction were observed according to the NCI-CTCAE v.3 criteria. No other moderate-to-severe adverse event was observed during vaccination (Table 2). After completion of 16 doses of peptide vaccine, enhanced CT on day 59 showed the progression of the target peritoneal lesions (sum of the tumor diameters increased from 39.3 to 44.2 mm) and the onset of multiple new lesions (Table 1). Serum CA19-9 decreased from 4,890.4 to 2,154.2 U/ml and to 2,029.9 U/ml 4 and 8 weeks after vaccinations, respectively. We decided to discontinue treatment. The patient developed ileus in November immediately after termination of therapy, and died of ileus in December 2008, 106 days after the initiation of protocol treatment. We failed to detect any immune response in this patient by PBMNC analysis.

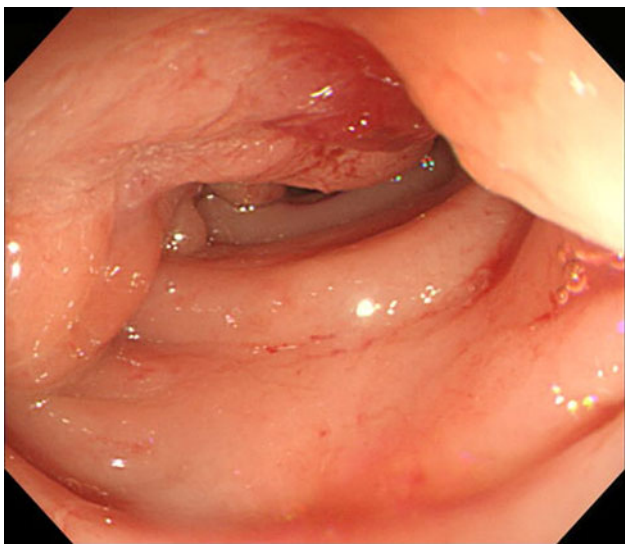

Fig. 1 Colonoscopic finding of rectosigmoid portion of the colon of Case 1 on day 8. Irregular surface mucosa indicates the transmural invasion of pelvic pancreatic cancer metastasis and fragile, easily bleeding mucosa, causes oozing

Table 2 Grade of adverse events (NCI CTCAE v.3.0)

	A*2402 protocol				A*0201 protocol	
Patient ID number	24021	24022	24023	24024	02011	02012
Blood/bone marrow						
Leukocytes	0	0	2	3	1	3
Neutrophils	0	0	2	3	1	3
Lymphopenia	2	0	3	3	0	2
Hemoglobin	2	2	0	0	2	0
Platelets	3	1	1	0	1	0
Metabolic/laboratory						
AST	2	0	0	0	0	0
ALT	1	0	0	0	0	0
GGT	2	0	0	0	0	0
Dermatology/skin						
Injection site reaction	0	0	1	0	1	1
Hemorrhage/bleeding						
Colon	0	0	2	0	0	0
Varices (esophageal)	0	0	0	0	3	0
%Dextramer-positive (% of dextramer-positive $CD8^+$ cells among $CD3^+$ lymphocyte-gated events)						
(Before vaccination)	NE	NE	NE	NE	0.38	0.05
(4 weeks)	NE	NE	NE	NE	8.89	2.74
(8 weeks)	NE	NE	NE	NE	14.8	3.12
ELISpot (spots/well)						
(4 weeks, with peptide)	NE	NE	NE	NE	604 ± 24.0	631 ± 39.2
(4 weeks, without peptide)	NE	NE	NE	NE	259 ± 20.5	91.7 ± 1.5
(8 weeks, with peptide)	NE	NE	NE	NE	661 ± 22.0	566 ± 38.6
(8 weeks, without peptide)	NE	NE	NE	NE	240 ± 17.7	103 ± 13.1

Adverse event grade were evaluated based on the National Cancer Institute Common Terminology Criteria of Adverse Event version 3. (NCI-CTCAE v.3); *AST* aspartyl aminotransferase; *ALT* alanyl aminotransferase; *GGT* gamma-glutamyl transpeptidase; *NE* not examined; *wks* weeks after vaccination; *ELISpot* enzyme-linked immunosorbent spot assay for the detection of peptide-specific interferon γ-release with or without peptide: number of the spots are indicated

Case 2

Two cases of advanced pancreatic cancer completed HLA-A*0201 protocol treatment (NCT00683085). The first case was a 50-year-old male who complained of jaundice in June 2008. A pancreatic head mass was observed as the cause of obstructive jaundice. He was initially diagnosed by contrast-enhanced CT scan as having advanced pancreatic cancer with multiple liver metastases, although an endoscopic biopsy of the pancreatic duct failed to detect malignant cells. He underwent endoscopic insertion of a mechanical stent from the common bile duct into the main pancreatic duct, and the jaundice resolved progressively. After the reduction of serum bilirubin concentration, he underwent 4 courses of GEM 700 mg/m^2 body surface area, but failed to respond to GEM. He was referred to our hospital in August 2008 to participate in the clinical trial.

The target lesions were the primary tumor and multiple liver metastases (sum of the diameters 84.0 mm). He underwent 6 courses of GEM with 16 doses of HLA-A*0201-restricted VEGFR1-derived peptide vaccine. After evaluation of the first course of protocol, he was diagnosed as PD based on the RECIST v.1.0 criteria (sum of the diameters was 118.8 mm after 16 doses of vaccine, and onset of multiple new lesions was observed by enhanced CT on day 55; see Table 1). A pancreatic head mass compressed his portal vein, splenic artery, and splenic vein. Portal hypertension and progress of collateral formation were indicated by enhanced CT. Observed toxicities were grade 1 injection site reaction, grade 1 leukopenia, grade 1 neutropenia, and grade 1 thrombocytopenia. No other systemic adverse event was observed (Table 2). In spite of failure to respond to therapy, the patient was willing to continue to be vaccinated, and a second course of treatment was initiated. After IRB approval confirming no disadvantage to vaccine continuation, he was vaccinated twice in October 2008. On day 7 of the second course of vaccination, he complained of upper abdominal pain with

sudden-onset nausea, and massive tarry stool thereafter. Upper gastrointestinal endoscopy revealed active bleeding from a lower esophageal varix and he then underwent endoscopic clipping. The etiology of the esophageal varices was considered to be portal hypertension due to compression of the portal vein by the progressive enlargement of the pancreatic tumor. We transfused 1,200 ml of concentrated red blood cells to ameliorate anemic symptoms. We decided to discontinue further vaccination and reported this episode to our IRB as a severe adverse event (SAE). He dropped out from our study, and further palliative therapy was initiated by daily oral administration of 100 mg S-1 (2 mg/kg) for 4 weeks followed by 2 weeks rest. He received 2 courses of S-1, and the last dose was given in December 2008. One early morning in January 2009, he felt nauseous and evacuated massive tarry stool. He was admitted to our hospital 3 days later, and an emergent upper gastrointestinal endoscopy showed oozing from enlarged esophageal varices (Fig. 2). Enlarged esophageal varices indicate impending portal hypertension with esophageal collateral formation. We discontinued further chemotherapy and initiated best supportive care (BSC) including palliative pericenthesis with sedative medication. To ameliorate tumor fever and abdominal discomfort, we administered 8 mg dexamethasone for 12 days, followed by 16 mg for 16 days. In April 2009 he died of hepatic failure, 242 days after the first vaccination. Autopsy findings indicated massive infiltration of the pancreatic head mass into the portal vein, splenic vein, and splenic artery. Massive ascites was yellow, but peritoneal dissemination was not recognized. The varix rupture due to portal hypertension was the possible cause of esophageal bleeding (Fig. 3). No lymphocyte infiltration around the tumor and varices was detected pathologically; however, post-mortem cryopreserved PBMNC analysis of this patient indicated that on day 29 and day 56 of vaccination, $CD8^+$ HLA-A*0201-restricted VEGFR1-specific dextramer-positive cells increased from 0.38% of all $CD3^+$ cells to 8.89 and to 14.80%, respectively (Fig. 4). Peptide-specific interferon-γ release was also increased from 604 ± 24.0 specific spots/well at responder/stimulator (R/S) ratio 1:1 (control without peptide 259 ± 20.5) on day 29 to 661 ± 22.0 specific spots/well on day 56 of

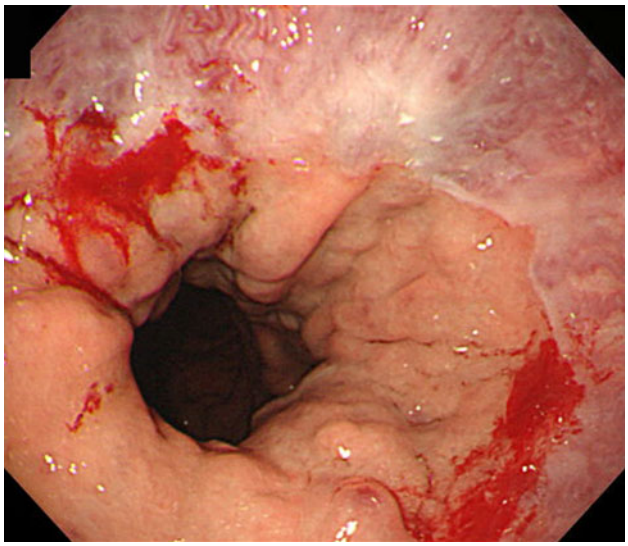

Fig. 2 Endoscopic finding of upper gastrointestinal tract of Case 2 on day 138. Endoscopic finding shows oozing from esophageal-cardiac junction varices

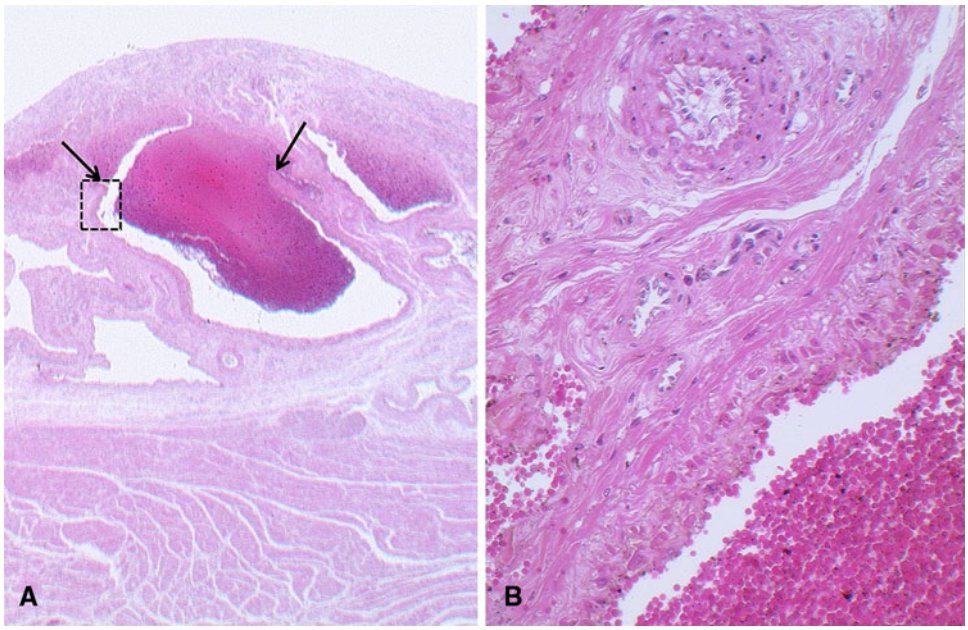

Fig. 3 Autopsy specimens of the esophageal varices. **a** Low-power view of esophageal varices. Ruptured portion is indicated by *arrows* (H&E staining). **b** Note that little mononuclear cell infiltration was detected in the varices wall (enlarged picture corresponds to *dashed box* in **a**)

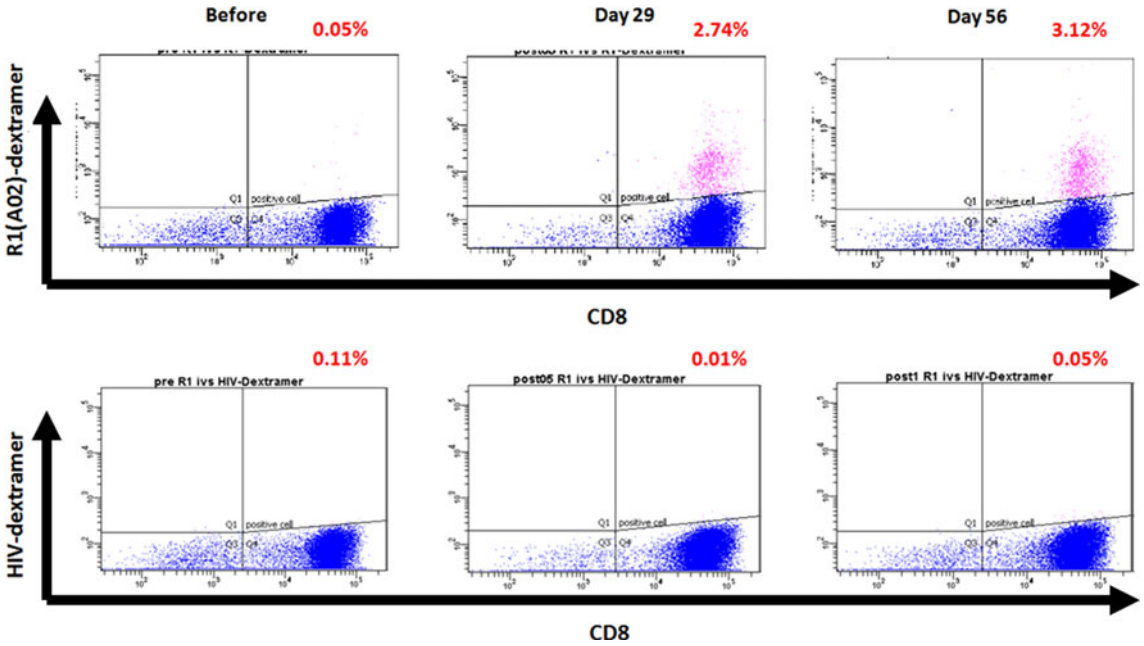

Fig. 4 HLA dextramer analysis. In Case 2, PBMNCs were collected and cryopreserved before vaccination, on day 29 and day 56. Cryopreserved PBMNCs were thawed and $CD4^+$ cells were depleted twice using Dynabeads $CD4^{TM}$ (Veritas Inc., Tokyo, Japan), then in vitro stimulated with HLA-A*0201-VEGFR1-peptide with T2 cell line for 3 days. After in vitro stimulation, patients' PBMNCs were incubated with HLA-A*0201-restricted VEGFR1-derived peptide (TLFWLLLTL)-dextramer complex with RPE probe (Immudex, Copenhagen, Denmark) or negative control dextramer, HLA-A*0201-restricted HIV-specific (ILKEPVHGV) dextramer−RPE. Percentages of dextramer-positive cells among $CD3^+CD8^+$ lymphocyte-gated cells were analyzed by FITC-conjugated anti-human CD8 mouse monoclonal antibody (clone RPA-T8), APC-conjugated anti-human CD3 mouse monoclonal antibody (clone; UCHT1), and FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA)

vaccinations at R/S ratio 1:1 (control without peptide 240 ± 17.7), respectively (Table 2). These results indicate that a certain immune response was observed, although with no clinical response and no pathologic lymphocyte infiltration.

Case 3

This is the second case to complete 16 doses of vaccine. The patient complained of anorexia in April 2008 and visited his home doctor in June 2008. His home doctor observed that the patient's tumor marker CA19-9 was higher than 17,000 U/ml and referred him to the local Red Cross Hospital for further evaluation. After a dynamic CT scan, he was diagnosed as having pancreatic cancer with liver and lung metastasis (CSIVb). He received eight courses of GEM combined with TS-1 (GEM 700 mg/m^2 intravenous biweekly on day 1 and day 15 with TS-1 50 mg/m^2 orally on day 1–14, every 4 weeks), then was switched to GEM single agent therapy (700 mg/m^2 biweekly) for two courses. A CT scan in November 2008, however, indicated PD on lung and liver metastasis. He was referred to our hospital for entry to our protocol and received 16 doses of HLA-A*0201-restricted VEGFR1-specific peptide vaccine in combination with GEM. The treatment was well tolerated and systemic adverse events were limited to grade 3 leukopenia, grade 3 neutropenia, and grade 2 lymphopenia, which were ascribed to GEM administration. Grade 1 local skin reaction was observed at injection sites. Clinical response was diagnosed as PD by enhanced CT on day 68 (sum of the target lesions on liver and lung had increased from 206.7 to 287.8 mm, an increase of more than 20%, with multiple new lesions), and the study was discontinued. He returned to the local hospital to receive BSC. Cryopreserved PBMNC analysis of this patient indicated that on day 29 and day 56 of vaccination, $CD8^+$ HLA-A*0201-restricted VEGFR1-specific dextramer-positive cells increased from 0.03% of all $CD3^+$ cells to 2.74 and to 3.12%, respectively (Table 2). However, peptide-specific interferon-γ release decreased from 631 ± 39.2 specific spots/well at responder/stimulator (R/S) ratio 1:1 (control without peptide 91.7 ± 1.5) on day 29 to 566 ± 38.6 specific spots/well on day 56 of vaccinations at R/S ratio 1:1 (control without peptide 103 ± 13.1), respectively (Table 2). In vitro results of dextramer analysis and ELISpot assay were not consistent with each other, as dextramer analysis was able to detect the effective specific immune responses, while ELISpot was not.

Discussion

The advantage of anti-angiogenic cancer therapy is theoretically maximal antitumor efficacy with minimal toxicities, because adult angiogenesis is limited to the local microenvironment of ischemia, inflammation and tumors [9]. Privileged selectivity compared to chemotherapy or radiotherapy ameliorates the detrimental adverse events and makes the therapy tolerable even in debilitated advanced cancer patients. In our experiments, no severe (>grade 3) systemic adverse events were observed other than grade 3 hematologic toxicities that may be ascribed to GEM administration. Thus anti-angiogenic peptide vaccination appears to be a relatively feasible treatment even in cases of advanced-stage pancreatic cancer. Our studies terminated early due to rapid disease progression and the advanced stage of the patients on entry. Considering time to progression (TTP) in all six cases in both protocols, median TTP was only 42 days (range 4–68 days, 24.5 days for HLA-A*2402 protocol, and 61.5 days for HLA-A*0201 protocol, respectively). This short duration of TTP indicated that advanced cases were selected for our protocol, and it was inappropriate to examine the immune and clinical responses as the immune system of the enrolled patients would be terminally debilitated. Further evaluation of patients at an earlier stage of pancreatic cancer is mandatory for precise estimation of feasibility and efficacy of the anti-angiogenic peptide vaccination strategy.

The anti-angiogenic strategy of cancer treatment is well tolerated and a relatively feasible treatment option compared to conventional chemoradiotherapy. Bevacizumab, a specific anti-VEGF humanized monoclonal antibody, causes only minor bleeding events and rarely causes life-threatening bleeding episodes [15–34, 37, 38].

Renal cell carcinoma (RCC) results from the inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene. RCC is sensitive to anti-angiogenic treatment because VEGF downstream signaling events are ubiquitously up-regulated. A randomized, double-blind, phase II trial of bevacizumab for metastatic RCC showed 8 cases with epistaxis in a high-dose group (10 mg/kg body surface area; $n = 39$) and 5 cases in a low-dose group (3 mg/kg body surface area; $n = 37$), in contrast to only 1 case in the placebo group ($n = 40$) [15]. A combination of the epidermal growth factor inhibitor erlotinib with bevacizumab for metastatic RCC caused more severe hemorrhagic events in a phase II trial. In the bevacizumab with erlotinib group ($n = 51$), 2 cases (3.9%) of grade 3 hemorrhagic events and 1 case (2.0%) of grade 4 hemorrhagic event were observed, whereas only 2 cases (3.8%) experienced grade 3 hemorrhage in the bevacizumab plus placebo group ($n = 53$) [16]. In a phase I study of bevacizumab with VEGFR-targeted tyrosine kinase inhibitor, sunitinib, 18 cases of hemorrhagic events of grade 1–4 including nose, oral, and rectal bleeding were observed among 25 participants with metastatic RCC [17]. Concomitant use of bevacizumab with combination chemotherapy causes more severe and more frequent bleeding compared to chemotherapy alone. Combination chemotherapy using irinotecan/fluorouracil/leucovorin (IFL) for metastatic colorectal cancer (CRC) complicates almost 3% of grade 3–4 bleeding events [18, 19]. An escalating dose of bevacizumab in combination with fluorouracil/leucovorin (FU/LV) for metastatic CRC complicates 46% (16 cases out of 35 participants) of epistaxis in the 5 mg/kg group, and 53% (17 cases out of 32 participants) of epistaxis in the 10 mg/kg group [20]. In the same study, GI bleeding events occurred in 2 out of 35 cases (6%) in the 5 mg/kg group and 5 out of 32 cases (16%) in the 10 mg/kg group. Generally, GI bleeding of grade 3–4 is observed in 3–5% of patients in phase II/III trials of CRC using FU/LV or an oxaliplatin/fluorouracil/leucovorin (FOLFOX-4)-based regimen in combination with bevacizumab [21–24]. Meta-analysis of multiple randomized trials for CRC indicated 2.7% of grade 3–4 bleeding events in chemotherapy combined with bevacizumab, and an odds ratio of 1.87 (95% confidence interval 1.10–3.16, $p = 0.02$) compared with chemotherapy alone [25]. Co-administration of bevacizumab did not significantly increase bleeding events with paclitaxel for metastatic breast cancer, nor with chemotherapy (docetaxel or pemetrexed single agent) or erlotinib for non-small-cell lung cancer (NSCLC) [26, 27]. However, grade 3–5 (fatal) pulmonary hemorrhage was reported with bevacizumab administration in combination with carboplatin/paclitaxel (PC) or cisplatin/GEM (CG) regimens for NSCLC [28–31].

In pancreatic cancer treatment using bevacizumab, GI bleeding is unavoidable. Ko et al. [32] reported 5 cases of GI bleeding (9.4%) among 53 participants treated with bevacizumab in combination with GEM/cisplatin. Kindler et al. [33] observed 16 cases (31%) of grade 1 bleeding and 1 case (2%) of grade 4 bleeding among 52 participants with advanced pancreatic cancer in a phase II trial of bevacizumab plus GEM. Crane et al. [34] described grade 3 bleeding from the area outside the radiation field when using bevacizumab in combination with radiation for advanced pancreatic cancer. Furthermore, serious hemorrhage may occur with the use of sorafenib and sunitinib for advanced or metastatic RCC [17, 35–37]. Recently, Kindler et al. [38] described the results of a phase III trial of GEM combined with and without bevacizumab. In their report, there was no increase in bleeding events in the GEM + bevacizumab group ($n = 277$) compared to the GEM + placebo group ($n = 263$). Grade 3–4 bleeding events were observed in 5% of the GEM + bevacizumab group and 4% of the GEM + placebo group ($p = 0.68$, not significant difference). There were 13 treatment-related

deaths, and five out of ten deaths on the experimental arm were potentially attributable to bevacizumab administration (one hemorrhage, two pulmonary embolism, and two perforations).

As described above, anti-angiogenic therapy combined with a chemotherapy regimen is associated with an increased risk of GI bleeding. The potential risk of GI bleeding associated with anti-angiogenic therapeutic intervention is theoretically induced by the inhibition of the wound healing process after vessel injury that may be caused coincidentally with angiogenesis/neovascularization. Ishizaki et al. [39] indicated that wound healing is not affected by the vaccination of HLA-A*0201-restricted VEGFR1-derived peptide in a human HLA-A2-transgenic murine model. In Case 1, the lower GI bleeding was ascribed to pancreatic cancer that directly invaded the rectosigmoid portion of the colon, as hematochezia occurred immediately after the first vaccination. Furthermore, an immune response in this case was not observed in vitro (data not shown). In Case 2, tumor obstruction of the portal vein and splenic vein caused bleeding from enlarged esophageal varices. Postmortem analysis of Case 2 failed to detect $CD8^+$ lymphocyte infiltration into tumors and esophageal varices. In vitro analysis of the patient-derived PBMNCs cryopreserved before the administration of dexamethasone indicated a gradual increase of HLA-A*0201-restricted VEGFR1-specific dextramer-positive lymphocyte population (Fig. 4) and peptide-specific IFN-γ-producing cells (Table 2) on day 29 and day 56 of vaccination, respectively. It is important that the positive dextramer assay in Case 3 was not consistent with the decreased immune response detected by ELISpot assay. Poor clinical responses were not correlated with promising in vitro immune responses from patients' PBMNCs in all cases, and discrepancy between in vitro assay and in vivo clinical response is a common phenomenon in immune therapy. Recently, Miyazawa et al. [41] reported a case of grade 3 duodenal hemorrhage in a phase I clinical trial using a peptide vaccine for human VEGF receptor type 2 (VEGFR2) in combination with GEM for 18 advanced pancreatic cancer patients. Careful exclusion of patients at risk of GI bleeding before the vaccination appears to be mandatory.

An unknown mechanism of anti-angiogenesis may contribute to the reduction of CA19-9 and the protection from tumor progression. After termination of peptide vaccination, Case 1 developed ileus and died shortly afterwards. A distinct decrease in CA19-9 was documented in this case. Case 2 survived 4 months on BSC after the termination of intensive therapy. Invasive ductal carcinoma usually shows rapid progression and durable survival supported only by BSC is rare among stage IV pancreatic cancer patients. Postmortem analysis indicated no evidence of peritoneal invasion. VEGFR1 enhances the migration and invasion of pancreatic cancer cell lines in vitro [14] and may contribute to the peritoneal dissemination by increasing vascular permeability and promoting tumor angiogenesis. It is possible that the VEGFR1 antagonization using a specific peptide may prevent peritoneal dissemination of pancreatic cancer.

Our studies were prematurely terminated because of the advanced stage of the disease in the enrolled patients on entry to the study and the short duration of TTP. However, the feasibility of the systemic administration of anti-angiogenic peptide vaccination is confirmed as systemic adverse events are limited to hematologic toxicities resulting from GEM administration. To evaluate the efficacy of this strategy, further evaluation with a sufficient number of patients is warranted.

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