MAP 432, the WHS 141, and the HNR 501 peptides enhanced CTLs reactive to the lck 246 peptide but also resulted in increased responses of CTLs reactive to both the lck 422 and the UBE 43 peptide, neither of which was administered into the patient (Fig. 1). After the third screening, this patient was vaccinated with the lck 246, the HNR 501, the lck 422, and the UBE 43 peptides, but this vaccination augmented CTLs reactive to the MAP 432 peptide. The same effect was found in the other 7 patients. However, no augmentation of peptide-specific CTLs was observed in patient EBG-007. As for patient EBG-104, the assay of CTL precursors before the sixth vaccination was not performed because the patient died after the fifth vaccination due to progression of disease.

Kinetic Assay of Cytotoxicity of the Second Regimen

Because of the limited availability of samples, we kinetically evaluated anti-tumor cytolytic activity of pre- and post-vaccination PBMCs in only four patients (Fig. 2). In patient EBG-001, CTL activity against SKG-I (HLA-A24$^+$) was augmented transiently after the vaccination, compared with that against QG56 (HLA-A24$^-$) but was subsequently diminished. In patient EBG-007, CTL activity against SKG-I increased after the sixth vaccination. In patient EBG-101, CTL activity against TOC-2 (HLA-A2$^+$) was transiently augmented compared with that against HLA-A2$^+$ PHA blasts. In patient EBG-102, CTL activity against TOC-2 was higher than that against HLA-A2- QG-56 throughout the peptide vaccination.

Serum IgG Specific to Peptides Administered

We also examined whether peptide-specific IgG could be detected in vaccinated patients (Table 5). Peptide-specific IgG was detected before vaccination in only patient, EBG-001. Although no peptide-specific IgG was detected in the other 9 patients before the peptide vaccination, peptide vaccination resulted in the induction of peptide-specific IgG in 9 of 10 pa-

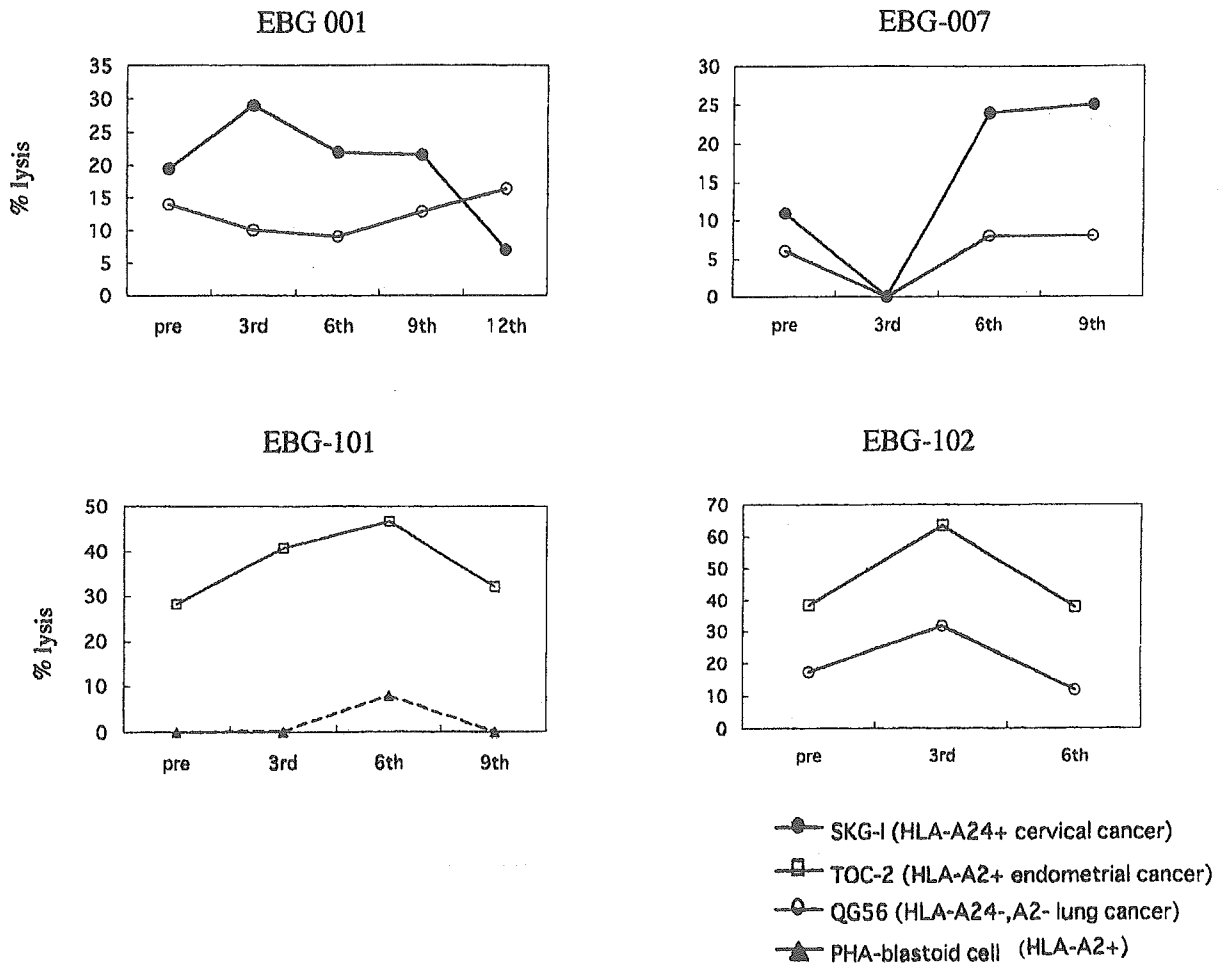


FIGURE 2. Kinetic analysis of cytotoxicity of vaccinated patients. Frozen pre- and post-vaccination (3rd, 6th, 9th, and 12th) PBMCs were thawed and incubated for 14 days with IL-2 alone without any peptides. A cytolytic assay against targets was carried out by a 6-hour ^{51}Cr-release assay at an E/T ratio of 40:1. Values are the means of triplicate assays. Patients EBG-001 and EBG-007 were positive for HLA-A24, and patients EBG-101 and EBG-102 were positive for HLA-A2.

tients. Representative results for patients EBG-001, EBG-002, EBG-004, EBG-006, and EBG-101 are shown in Figure 3a. In patients EBG-001 and EBG-002, peptide-specific IgG was detected after the ninth vaccination. In patients EBG-004, EBG-006, and EBG-101, 6 vaccinations were sufficient to elicit peptide-specific IgG. As shown in Figure 3b, peptide-specific IgG in the plasma of patient EBG-001 was absorbed by culturing on peptide-coated plates in an antigen-specific manner. Namely, the reactivity to lck 208 or SART3 109 peptide was diminished by culturing on plates coated with the relevant peptides, but not on those coated with irrelevant peptides.

DTH Skin Test

No DTH reaction against peptides was observed before vaccination in any patients (Table 5). Peptide-specific DTH reactions were observed in three patients after the vaccination in the second regimen. In the first patient, EBG-003, DTH reaction to each of the SART2 899, the SART2 93, and the SART3 315 peptides was observed after the peptide vaccination. Another patient, EBG-004, exhibited the DTH reaction against the SART2 161, CypB 91, and SART3 315 peptides, while patient EBG-102 exhibited the DTH reaction against the SART3 302 and lck 422 peptides after the second and third vaccinations, respectively.

Clinical Responses

As shown in Table 3, the first regimen failed to induce any clinical response in the four patients, all of whom showed progressive disease. In the second regimen, among the seven patients who had measurable disease at entry, two patients (EBG-001 and EBG-101) and two patients (EBG-004 and EBG-103) showed PR and stable disease, respectively (Tables 3 and 5). As shown in Figure 4a, tumor regression was observed in patient EBG-001, who had para-aortic lymph node metastasis, and that lesion was reduced 42% between the tenth and 17th vaccinations, with the level of carcinoembryonic an-

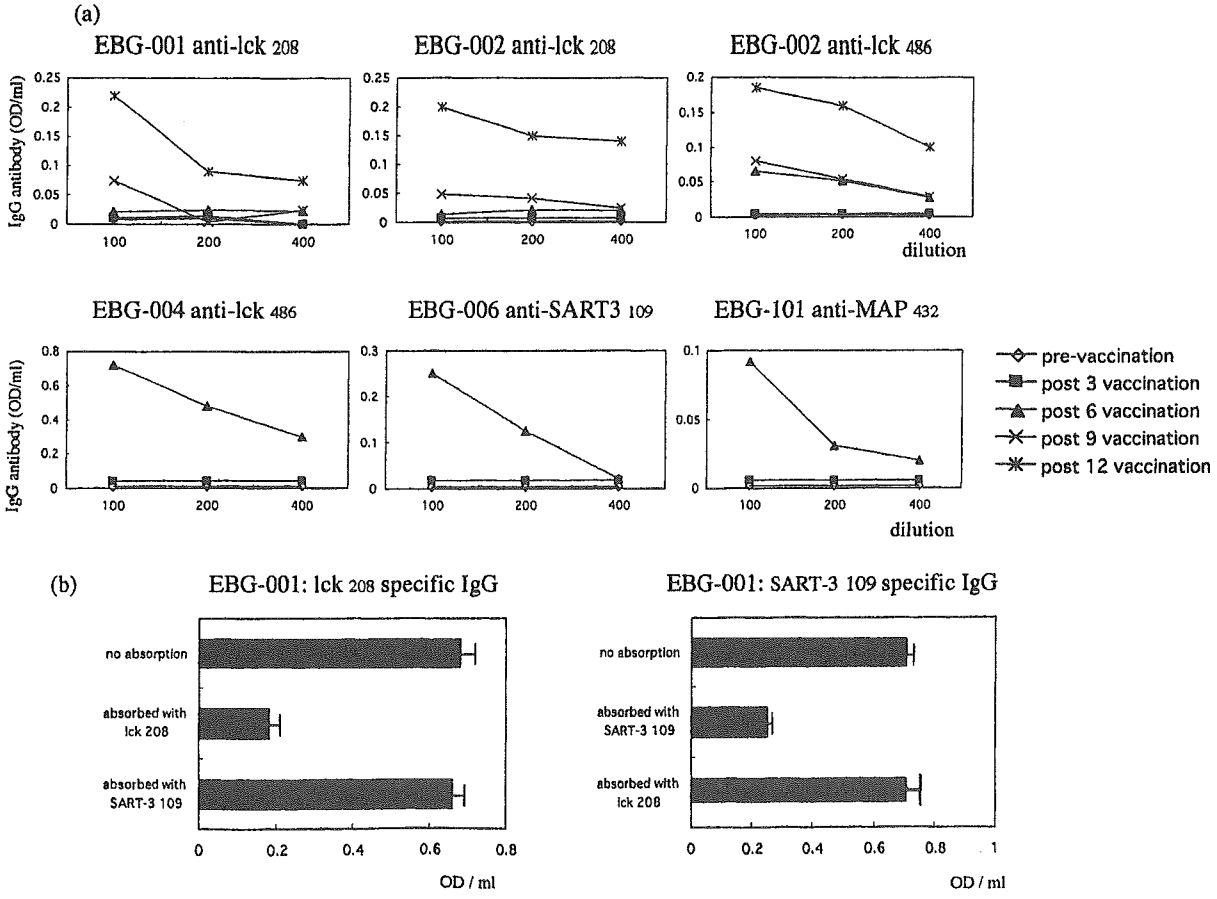
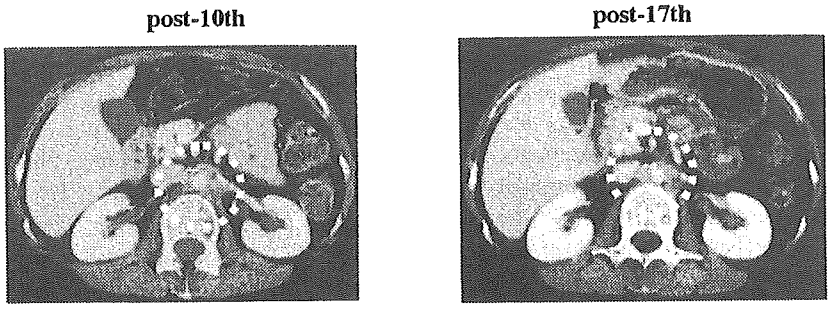
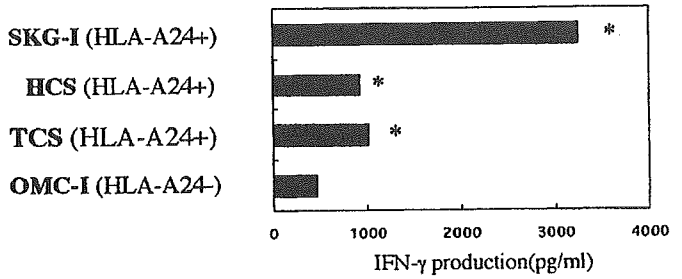


FIGURE 3. Serum IgG reactive to vaccinated peptides. **a:** The levels of peptide-specific IgG of pre- and post-vaccination plasma from 5 patients were determined by ELISA. **b:** Plasma from patient EBG-001 after the 12th peptide vaccination was cultured with the indicated peptide-coated plates. Thereafter, the levels of IgG reactive to relevant peptides in the resultant samples were determined by ELISA.

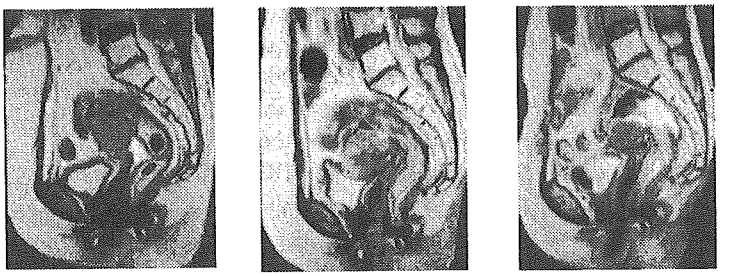
(a) [EBG-001]



Shrinkage of tumor 42%
Level of CEA(ng/ml) 188 105

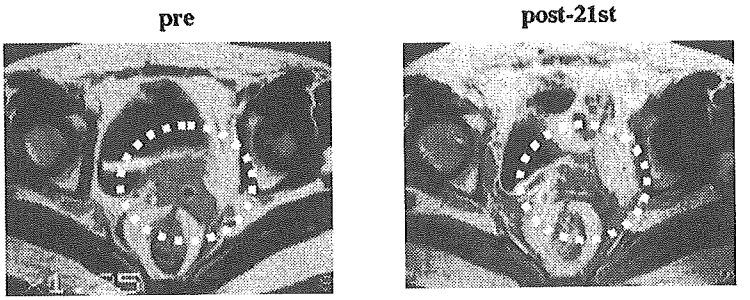


(b) [EBG-101]



Shrinkage of tumor 26% 48%
Level of SCC (ng/ml) 273 289 36

(c) [EBG-103]



Shrinkage of tumor 13%

tigen decreasing from 207 to 105. This patient received irradiation to the para-aortic lymph node metastases two months before the peptide vaccination but did not receive irradiation to the primary lesion. This patient was judged as PR four months after the peptide vaccination. In addition, in vitro cultured inguinal LN cells, which were draining from the vaccination site, produced a higher level of IFN-γ in response to HLA-A24$^+$ tumor cells than in response to HLA-A24$^-$ tumor cells. Patient EBG-101, with recurrent cervical cancer invading to uterine body, also showed objective tumor shrinkage. The MRI results of patient EBG-101 at prevaccination and after the fifth and 10th vaccinations are shown in Figure 4b. This patient was diagnosed as showing a PR for 10 months. In this patient, the levels of squamous cell carcinoma (SCC)-related antigen and carcinoembryonic antigen decreased from 289 and 13.3 to 36 and 6.6, respectively. This patient received irradiation to the lesion 5 years before the peptide vaccination. Patient EBG-103 was not judged as PR because the evaluated longest tumor diameter showed a 13% reduction, although the tumor invading to the parametrium drastically shrunk after the peptide vaccination (Fig. 4c). This patient received irradiation to the lesion 2 months before the peptide vaccination. Patient EBG-102 showed progressive disease 3 months after the vaccination, but the levels of tumor markers were significantly decreased: the levels of CA125 and CA19-9 decreased from 24,000 and 113 to 17,000 and 29.3, respectively. Three patients (EBG-002, EBG-007, and EBG-104) were diagnosed with progressive disease at two months after the vaccination. Although the remaining three patients (EBG-003, EBG-004, and EBG-006) had no measurable disease at entry, they were enrolled in this study because of their high risk of recurrence, and they agreed to enter to the trial for purpose of prophylaxis. Patient EBG-004 showed stable disease for eight months, but patients EBG-003 and EBG-006 were diagnosed with progressive disease. In most cases, patients received the peptide vaccination of the second regimen as outpatients, and the performance status remained good throughout the treatment period.

DISCUSSION

We previously identified a panel of antigenic peptides capable of inducing tumor-reactive CTLs in HLA-A24$^+$ or HLA-A2$^+$ patients.[6–16] In a subsequent study, we vaccinated some of these peptides into cancer patients, but induction of cellular responses to either peptides or cancer cells was insufficient in the postvaccination PBMCs.[23] In this study, we conducted two different regimens for patients with gynecologic cancers. In the first regimen, 4 HLA-A24$^+$ patients were vaccinated with either SART2-derived or ART4-derived peptides, which were predesignated before the vaccination. Although the vaccination protocol was completed safely, no objective response was observed. However, in the second regimen, 6 HLA-A24$^+$ and 4 HLA-A2$^+$ patients were vaccinated with peptides to which CTL precursors were preexisting before vaccination. Increased cellular responses to the vaccinated or nonvaccinated peptides were observed in the postvaccination PBMCs of 7 of 10 patients tested. In addition, increased humoral responses to the vaccinated peptides were observed in the postvaccination plasma of nine of ten patients tested. Three patients with cervical cancer showed objective tumor regression. These lines of evidence indicate that the second, evidence-based peptide vaccination is feasible and superior to the first, predesignated peptide vaccination for treatment of patients with recurrent gynecologic cancers, especially cervical cancer.

In the evidence-based vaccination, the efficacy of the peptide vaccination was evaluated by several methods. We evaluated the reactivity of CTLs using ELISA for IFN-γ and found that peptide-specific IFN-γ-producing CTLs could be induced by the peptide vaccination with higher incidence. We applied a cytolytic assay and found that the evidence-based peptide vaccination could enhance the cytotoxicity of CTLs, although only a small number of patients were examined in this study. We also measured the levels of IgG reactive to the administered peptides. The results showed that peptide-specific IgG was elicited in most cases. We checked the DTH reaction to administered peptides but found that DTH reactions were induced in only 3 of 10 patients. The results from these different methods did not appear to be correlated. However, two assays (ie, the assay of peptide-specific IFN-γ production and that of peptide-specific IgG induction) appeared to be useful in evaluating the efficacy of peptide vaccination, since these two responses, but not the DTH response, were observed in 3 cases (EBG-001, EBG-101, and EBG-103) that showed objective tumor regression or long stable disease. Cytolytic activity against cancer cells was also enhanced in patients EBG-001 and EBG-101. Although DTH response is generally considered useful in monitoring peptide-specific immune response, this was not the case in the present trial. Although recent reports indicate that ELISPOT assay is a useful method to monitor peptide-specific T cell responses,[24,25] we did not carry out

FIGURE 4. Tumor regression in 3 patients. **a:** The size of the para-aortic LN with metastasis of patient EBG-001 was evaluated using CT scan. The level of carcinoembryonic antigen in serum was measured after the 10th and 17th vaccination. Inguinal LN cells with metastasis of patient EBG-001 were cultured with IL-2 (100 U/mL) for 14 days, and IFN-γ production in response to 4 kinds of gynecologic cancer cell lines was determined by ELISA. *$P < 0.05$ statistically significant compared with a control. **b:** Patient EBG-101 was kinetically evaluated for the size of tumor mass using MRI. The levels of SCC in the serum were kinetically measured. **c:** Patient EBG-103 was kinetically evaluated for the size of tumor mass using MRI.

this assay in this study. Further studies will be needed to determine which methods are the most useful in monitoring peptide-specific T cell responses.

In the present study, we assessed peptide-specific CTL responses based on a classification consisting of two parameters: the *P* value and IFN-γ release. The main reason for using this classification was that the level of IFN-γ produced by peptide-specific CTLs varied among quadruplicate wells. It is possible that one well may have contained peptide-specific CTL precursors, whereas another may have contained none. Another reason was that we had to examine the presence of CTL precursors specific to 14 or 16 different kinds of peptides using the limited number of PBMCs from cancer patients. We considered that each well should be individually estimated to screen for the presence of peptide-specific CTL precursors.

All of the peptides used in this study were derived from nonmutated self-antigens involved in cellular proliferation, whereas the most common adverse events of this clinical study were inflammatory reactions at the vaccination site. Fever was also frequently observed. One patient (EBG-101) developed rectal bleeding after the 6th vaccination, but obvious correlation to the peptide vaccination was unclear because this patient had radiation colitis in the rectum before entry into this trial. However, because this patient showed augmented cellular responses after the peptide vaccination, the possibility that the rectal bleeding might have been triggered by an enhanced immune response cannot be excluded.

Because three cervical patients who showed objective clinical responses had received irradiation therapy, there remains the possibility that their responses were caused by irradiation. However, the time intervals between irradiation and the peptide vaccination of patients EBG-101, EBG-102, and EBG-103, were 2 months, 5 years, and 2 months, respectively. In addition, the peptide vaccination was started at least 2 months after radiation therapy, whereas clinical responses had been observed several months after the peptide vaccination and had been continued for more than 10 months. Based on these lines of evidence, we consider that the clinical responses in these three cervical cancer patients were not due to radiation therapy but to the peptide vaccination.

In the evidence-based regimen, peptide-specific IgG was induced in most cases after the peptide vaccination, and clinical responses seemed to be associated with the induction of IgG to the administered peptides. At the present time, we have no idea about what roles IgG plays in the anti-tumor response in cancer patients, or about the results of vaccination-associated IgG induction specific to administered peptides. Peptide-specific IgG might show a direct or indirect anti-tumor effect in cooperation with cellular immunity. CD4$^+$ T cells might participate in the induction of peptide-specific IgG, since in vivo generation of antigen-specific IgG generally requires a cytokine from helper T cells.[26] Information regarding the roles of peptide-specific IgG in peptide-vaccinated patients may contribute to the design of more effective anti-tumor immunotherapy.

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Humoral Responses to Peptides Correlate with Overall Survival in Advanced Cancer Patients Vaccinated with Peptides Based on Pre-existing, Peptide-Specific Cellular Responses

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ABSTRACT

Purpose: **The aim of this study is to find a laboratory marker for overall survival in advanced cancer patients who were vaccinated with peptides based on pre-existing, peptide-specific CTL precursors in the circulation.**

Experimental Design: **A group of 113 patients with advanced cancer (28 colorectal, 22 prostate, 15 lung, 14 gastric, and 34 other cancers) was enrolled in a Phase I clinical study of peptide vaccination in which peptide-specific CTL precursors of prevaccination peripheral blood mononuclear cells were measured, followed by vaccination with these peptides (maximum of four). For cellular responses, pre and postvaccination (sixth) peripheral blood mononuclear cells were provided for measurement of both peptide-specific CTL precursors by IFN-γ release assay and tumor reactivity by ^{51}Cr release assay. Delayed type hypersensitivity was also measured. For humoral response, pre and postvaccination (sixth) sera were provided for measurement of peptide-reactive IgG by an ELISA.**

Results: **The median survival time and 1-year survival rate of the total cases were 346 ± 64.9 days and 44.6%, respectively, and those of patients vaccinated more than six times (n = 91) were 409 ± 15 days and 54.4%, respectively. In these 91 patients, the overall survival of patients whose sera showed increased levels of peptide-reactive IgG (n = 60) was significantly more prolonged (P = 0.0003) than that of patients whose sera did not (n = 31), whereas none of cellular responses correlated with overall survival.**

Conclusions: **Peptide-specific IgG in postvaccination sera could be a suitable laboratory maker for the prediction of prolonged survival in advanced cancer patients vaccinated with peptides based on pre-existing CTL precursors.**

INTRODUCTION

Recent advances in tumor immunology have allowed the identification of a number of antigens and epitopic peptides capable of inducing tumor-reactive CTLs (1–14). Some of these peptides were used for clinical trials, but these initial trials obtained rare clinical responses, as well as dim levels of immune responses to peptides (15–20). One reason for this failure could be an insufficient induction of antitumor responses in these regimens, in which peptide-specific memory T cells were not measured in prevaccination peripheral blood mononuclear cells (PBMCs). Subsequently, we conducted Phase I clinical trials of peptide vaccination in which cancer patients received peptides (a maximum of four) based on information regarding pre-existing, peptide-specific CTL precursors in the circulation (21–25). The other reason for failure might in part be attributable to a lack of an appropriate laboratory marker either to measure immune responses or to predict clinical responses. Regardless of the extensive studies, there are few reproducible and appropriate laboratory markers for prediction of clinical benefits in recently developing peptide-based therapies (15–25) or in the other types of immunotherapies (26–28). In this study, we investigated the correlation of clinical benefits and immune responses to peptides in HLA-A24-positive or -A2-positive cancer patients who were vaccinated with these CTL-directed peptides and reported that humoral responses to peptides correlated with overall survival.

MATERIALS AND METHODS

Trial Eligibility. The ethical review boards of the Kurume University School of Medicine and the Hokkaido University School of Medicine approved the study protocol. Complete written informed consent was obtained from all patients at the time of enrollment. According to the protocol, patients were required to be HLA-A24 positive and/or HLA-A2 positive, have a histologically confirmed lesion of a malignant tumor, have been untreated for ≥4 weeks before the study, and have an Eastern Cooperative Oncology Group performance status of 0–2. Eligibility criteria included an age from 20 to 85 years, a

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creatinine level <1.4 mg/dl, a bilirubin level <1.5 mg/dl, a platelet count of >100,000/mm^3, hemoglobin of >8 grams/dl, and total WBC count of >3000/μm^3. Hepatitis B and C antigens were required to be negative. No patient had received any concurrent treatments, steroids, or any other immunosuppressive drugs for 4 weeks before the initial vaccination. This clinical study was carried out from November 2000 through November 2002.

Peptides and Selection for Vaccination. The peptides used in the present study were prepared under conditions of Good Manufacturing Practice by the Multiple Peptide System (San Diego, CA). The peptide sequences are shown in Table 2. These peptides have the ability to induce HLA-A24- or -A2-restricted and tumor-specific CTL activity in the PBMCs of cancer patients and were frequently expressed on various tumor cell lines (5–14). These peptides were dissolved and stored at −80°C. Stock solutions were diluted with saline just before use. For the peptide screening, prevaccination PBMCs were provided for assays of peptide-specific CTL precursors using methods reported previously (29). Peptide-specific IFN-γ production was calculated by subtraction of IFN-γ production of the peptide-stimulated PBMCs in response to a negative control (HIV peptide) from that in response to a corresponding peptide in quadricate assays, and a two-tailed Student's t test was used for the statistical analyses. As reported previously (21–25), positive wells were evaluated in the following order: (*a*) criteria Ar, the peptide-specific IFN-γ production was ≥500 pg/ml, and P was <0.1; (*b*) criteria A, the production was ≥50 pg/ml, and P was <0.05; (*c*) criteria B, 25 ≤ the production <50 pg/ml, and P was <0.05; (*d*) criteria C, the production was ≥50 pg/ml and $0.05 \leq P < 0.1$; and (*e*) criteria D, the production was >100 pg/dl and $0.1 \leq P < 0.2$. According to the results, up to four positive peptides were selected for each patient and were vaccinated as the CTL precursor-oriented peptide vaccine, if an immediate type hypersensitivity reaction against each peptide was not seen in a skin test performed before vaccination. The screening of peptide-specific CTL precursors was also performed by the same method after the sixth vaccination to evaluate the *in vivo* cellular responses to the peptides. Cellular responses to tumor cells in a HLA-A24- or -A2-restricted manner in pre and postvaccination (sixth) PBMCs were measured using a standard ^{51}Cr-release assay whose methods were described elsewhere (19, 20).

Clinical Protocol. Skin tests were performed by intradermal injection of 10 μg of each peptide using a tuberculin syringe and a 26-gauge needle. Saline was used as a negative control for assessment of hypersensitivity. Immediate and delayed type hypersensitivity (DTH) reactions were determined at 20 min and 24 h after the skin test, respectively. At least 5 mm of induration or 10 mm of erythema read 24 h after injection were needed to score the skin test as positive. If immediate type hypersensitivity was negative, the peptide was vaccinated into the patients' s.c. tissue in the site near each tumor's regional lymph nodes, *e.g.*, the upper arm in cases with lung cancer, lateral abdominal wall in cases with gastric cancer, or anterior thigh with the other cancers. Two milliliters of the peptide, which was supplied in vials containing 2 mg/ml sterile solution, were mixed with an equal volume of incomplete Freund's adjuvant (Montanide ISA-51; Seppic, Paris, France) and emulsified in 5-ml sterilized syringes. Three milliliters of each prepared peptide emulsion (maximum of four peptides at one vaccination) were injected s.c. in individual site three times every 2 weeks. For patients showing a favorable clinical course, the vaccinations were continued every 2–4 weeks with informed consent from each patient.

Detection of Serum IgG Levels. An ELISA was used to detect the serum IgG levels specific to the administered peptides, as reported previously (20–25). Briefly, 100 μl/well serum samples diluted with 0.05% Tween 20-Block Ace were added to the peptide (20 μg/well)-immobilized plate, after which the plate was blocked with Block Ace (Yukijirushi, Tokyo, Japan) and washed. After a 2-h incubation, the plate was washed and further incubated for 2 h with a 1:1000-diluted rabbit antihuman IgG (DAKO, Glostrup, Denmark). The plate was washed, and 100 μl of 1:100-diluted goat antirabbit immunoglobulin-conjugated horseradish peroxidase-dextran polymer (EnVision; DAKO) were then added to each well; the plate was incubated for 40 min. After washing, 100 μl/well tetramethyl-benzidine substrate solution (KPL, Guildford, United Kingdom) were added, and the reaction was stopped by the addition of 1 M phosphoric acid. To estimate peptide-specific IgG levels, the absorbance values of each sample were compared with those of serially diluted standard samples, and the values were shown as absorbance. The cutoff value of optimal density (OD) was determined as 0.02 at a serum dilution of 1:100, because the mean of the OD in response to an HIV peptide taken as a negative control was <0.02, as reported previously (21–25). Positive responses were judged by the two criteria. The first criterion is the case if the prevaccination serum showed no reactions, and the postvaccination serum showed at least significant levels of IgG (>0.02 of net OD value at a serum dilution of 1:100) specific to the vaccinated peptides. The other criterion is the case if an OD value of the postvaccination serum showed ≥2-fold increase at a serum dilution of 1:100 than that of the prevaccination serum.

Evaluation of Clinical Responses and Statistical Analysis. All known sites of disease were evaluated by computed tomography scan or X-ray examination. Patients were assigned a response category according to the Response Evaluation Criteria In Solid Tumors (RECIST criteria), the revised version of the WHO criteria published in the WHO Handbook for reporting results of cancer treatment (Geneva, 1979), June 1999 (Final). For prostate cancer patients without measurable lesions, serum prostate-specific antigen levels were used as a marker for evaluation, as reported previously (23, 25). Overall survival was evaluated from the entry date of these clinical trials, regardless of peptide vaccinations after Phase I trials, and analyzed to investigate correlation between clinical benefits and immune responses. Kaplan-Meier curves were described, and survivals were compared using the Log-rank test.

RESULTS

Patients' Characteristics. A group of 113 patients with advanced malignant tumors was enrolled in this vaccination regimen. The types of cancer included the following: (*a*) colorectal cancer (HLA-A24: n = 22, HLA-A2: n = 6); (*b*) prostate cancer (n = 12, 10); (*c*) lung cancer (n = 10, 5); (*d*) gastric

Table 1 Patients characteristics and median survival times

Sort of cancer	n	Age (range)	Male/Female	HLA-A24/-A02	PS[a] 0/1/2	MST ± SE (days)
Colorectal cancer	28	57.5 (27–78)	20/8	22/6	13/12/3	273 ± 70.9
Prostate cancer	22	68.1 (50–85)	22/0	12/10	19/3/0	601 ± 84.1
Lung cancer	15	65.7 (58–74)	9/6	10/5	9/2/4	668 ± 201.2
Gastric cancer	14	64.7 (49–78)	12/2	12/2	3/8/3	139 ± 48.4
Melanoma	7	60.7 (30–76)	4/3	5/2	5/2/0	353 ± 82.5
Cervical cancer	6	50.8 (32–67)	0/6	4/2	6/0/0	324 ± 157.3
Ovarian cancer	3	55.0 (49–59)	0/3	1/2	2/1/0	522 ± 344.6
Breast cancer	3	46.3 (38–58)	0/3	1/2	1/2/0	
Esophageal cancer	3	63.7 (59–70)	2/1	1/2	1/0/2	85 ± 33.1
Uterine cancer	2	61.5 (56–67)	0/2	2/0	2/0/0	415 ± 293.4
Pancreatic cancer	2	61.5 (60–63)	1/1	2/0	1/1/0	165 ± 95.8
Leiomyosarcoma	2	57.5 (54–61)	0/2	2/0	1/0/1	61 ± 43.1
Thyroid cancer	1	62	0/1	1/0	1/0/0	
Chronic lymphocytic leukemia	1	77	1/0	1/0	0/1/0	
Bladder cancer	1	53	1/0	1/0	1/0/0	199
Renal cell carcinoma	1	39	1/0	1/0	1/0/0	
Periurethral cancer	1	55	0/1	0/1	1/0/0	
Seminoma	1	23	1/0	0/1	1/0/0	409
Total	113	61.0 (23–85)	74/39	78/35	68/32/13	346 ± 64.9

[a] PS, performance status; MST, median survival time.

cancer (n = 12, 2); (e) melanoma (n = 5, 2); (f) cervical cancer (n = 4, 2); (g) ovarian cancer (n = 1, 2); (h) breast cancer (n = 1, 2); (i) esophageal cancer (n = 1, 2); (j) uterine cancer (n = 2, 0); (k) pancreatic cancer (n = 2, 0); (l) liomyosarcoma (n = 2, 0); (m) thyroid cancer (n = 1, 0); (n) chronic lymphocytic leukemia (n = 1, 0); (o) bladder cancer (n = 1, 0); (p) renal cell carcinoma (n = 1, 0); (q) periurethral cancer (n = 0, 1); and (r) seminoma (n = 0, 1; Table 1). The average patient age was 61 years (range: 23–85). Patients' performance status evaluated by Eastern Cooperative Oncology Group criteria was 0 (n = 68), 1 (n = 32), and 2 (n = 13). All of the patients showed failure to respond to chemotherapy, hormonal therapy, and/or radiotherapy with clinical stage IV or recurrence. Details regarding the characteristics of patients with each respective type of cancer were described in the other studies (21–24), currently in press (25) or under submission.

Vaccinated Peptides and Immune Responses. The number of patients receiving four, three, two, or one peptide without immediate type hypersensitivity were 50, 19, 8, or 1 in HLA-A24$^+$ patients, respectively, and 21, 9, 3, or 2 in HLA-A2$^+$ patients, respectively. CTL precursors reacting to peptides were detected in prevaccination PBMCs for vaccination; the frequency of vaccinated peptides is given in Table 2. The most frequently used peptide was the $SART3_{109}$ (38 of 78 cases), followed by the lck_{208} (31 cases) in HLA-A24$^+$ patients. In HLA-A2$^+$ patients, the most frequently used peptide was the MAP_{294} (15 of 35 cases), followed by the MAP_{432} (14 cases). CTL activity was evaluated in postvaccination (sixth) PBMC to evaluate cellular immune responses to the vaccinated peptides. Increased cellular responses were most frequently observed when the $SART3_{315}$ peptide was vaccinated (9 of 22 cases, 41%) followed by the $SART3_{109}$ peptide (12 of 31 cases, 39%). Detailed results for each case have been reported elsewhere (21–25) and are summarized in Table 2. CTL activity to HLA-class I-restricted tumor cells was measured by the standard ^{51}Cr-release assay in pre and postvaccination (third and sixth) PBMCs. Sixteen of 76 cases tested (21%) showed increased HLA-class I-restricted cytotoxicity. DTH response at the site of a skin test during the first to sixth vaccination was most frequently observed when the $SART3_{315}$ peptide was vaccinated (9 of 26 cases of HLA-A24$^+$ patients). DTH response was most frequently observed in HLA-A2$^+$ patients vaccinated with the lck_{422} peptide (6 of 12 cases), a summary of which is presented in Table 2. Humoral immune responses to the vaccinated peptides were simultaneously measured in both pre and postvaccination (third and sixth) sera. Increased levels of IgG antibodies reactive to peptides were most frequently observed when the $SART3_{109}$ was vaccinated (19 of 37 cases), as summarized in Table 2. It is of note that the UBE_{43} peptide induced humoral immune responses in all five cases tested. Detailed results and the criteria of increased immune responses for each case have been reported elsewhere (21–25) and are under submission as separate studies.

Clinical Responses and Prognostic Marker Analysis. Of 113 cases, 5 cases showed partial response, 2 cases showed minor response, and the remaining 106 cases showed progressive disease. A median survival time of 113 cases was 346 ± 64.9 (±SE) days, and a 1-year survival rate was 44.6% (Fig. 1*A*). Twenty-two cases could not achieve one cycle of vaccination (six times) because of the rapid progression of tumors, whereas the remaining 91 cases received more than six vaccinations. The median survival time and 1-year survival rate of these 91 cases were 409 ± 15 days and 54.4%, respectively (Fig. 1*B*). In patients undergoing more than six vaccinations (n = 91), 60 cases had detectably increased levels of peptide-specific IgG antibody in their postvaccination sera against at least one peptide of at maximum four vaccination peptides, whereas the remaining 31 cases did not (Fig. 1*C*). Forty-two among 90 cases tested showed increased CTL activity response to at least one peptide of at maximum four vaccination peptides (Fig. 1*D*), and 16 of 73 cases tested showed increased CTL activity of HLA-class I-restricted cytotoxicity against tumor

Table 2 Vaccinated peptide and immune responses

Peptide name	Sequence	No. of vaccinated patients	Increased immune reactions to peptide[a]		
			CTL activity	IgG antibody	DTH[b] response
HLA-A24-binding					
SART1 690	EYRGFTQDF	20	1 /17 (6%)	2 /19 (11%)	2 /20 (10%)
SART2 93	DYSARWNEI	19	3 /15 (20%)	4 /17 (24%)	0 /19
SART2 161	AYDFLYNYL	21	2 /16 (13%)	1 /18 (6%)	2 /21 (10%)
SART2 899	SYTRLFLIL	13	0 /7	2 /11 (18%)	3 /13 (23%)
SART3 109	VYDYNCHVDL	38	12 /31 (39%)	19 /37 (51%)	4 /38 (11%)
SART3 315	AYIDFEMKI	26	9 /22 (41%)	6 /25 (24%)	9 /26 (35%)
CypB84	KFHRVIKDF	4	0 /3	2 /3 (67%)	0 /4
CypB91	DFMIQGGDF	19	0 /17	1 /17 (6%)	1 /19 (5%)
Ick208	HYTNASDGL	31	7 /25 (28%)	9 /29 (31%)	6 /31 (19%)
Ick486	TFDYLRSVL	25	4 /21 (19%)	7 /22 (32%)	6 /25 (24%)
Ick488	DYLRSVLEDF	26	3 /21 (14%)	1 /24 (4%)	5 /26 (19%)
ART1 170	EYCLKFTKL	9	0 /7	2 /7 (29%)	0 /9
ART4 13	AFLRHAAL	4	0 /1	0 /2	0 /4
ART4 75	DYPSLSATDI	19	1 /16 (6%)	0 /15	1 /19 (5%)
HLA-A02-binding					
SART3 302	LLQAEAPRL	6	0 /5	0 /5	0 /6
SART3 309	RLAEYQAYI	9	0 /7	1 /7 (14%)	0 /9
CypB129	KLKHYGPGWV	6	0 /6	2 /6 (33%)	1 /6 (17%)
CypB172	VLEGMEVV	6	1 /4 (25%)	3 /5 (60%)	1 /6 (17%)
Ick246	KLVERLGAA	7	2 /7 (29%)	6 /7 (86%)	0 /7
Ick422	DVWSFGILL	12	3 /11 (27%)	0 /10	6 /12 (50%)
MAP294	GLLFLHTRT	15	1 /13 (8%)	8 /15 (53%)	7 /15 (47%)
MAP432	DLLSHAFFA	14	3 /11 (27%)	5 /13 (38%)	4 /14 (29%)
WHSC103	ASLDSDPWV	8	0 /6	1 /8 (13%)	1 /8 (13%)
WHSC141	ILGELREKV	8	1 /4 (25%)	2 /5 (40%)	0 /8
UBE43	RLQEWCSVI	6	1 /5 (20%)	5 /5 (100%)	2 /6 (33%)
UBE85	LIADFLSGL	2	1 /2 (50%)	1 /2 (50%)	0 /2
UBE208	ILPRKHHRI	1	0 /1	1 /1 (100%)	0 /1
HNRPL140	ALVEFEDVL	5	0 /5	0 /5	0 /5
HNRPL501	NVLHFFNAPL	13	1 /11 (9%)	3 /13 (23%)	2 /13 (15%)
EIF51	RIIYDRKFL	1	0 /1	1 /1 (100%)	0 /1

[a] No. of tested cases and % positive in parentheses are shown.
[b] DTH, delayed-type hypersensitivity.

cells (Fig. 1*E*) in their postvaccination PBMCs. Thirty-four of 91 cases showed DTH response to at least one peptide among at maximum four vaccination peptides until the sixth vaccination (Fig. 1*F*). None of the cellular responses (peptide-specific CTL precursors, tumor-reactive CTL activity, or DTH reaction) correlated with overall survival. In contrast, the overall survival of patients whose sera showed increased levels of peptide-reactive IgG antibodies ($n = 60$) was more significantly prolonged ($P = 0.0003$) than that of patients whose sera did not show such increased levels ($n = 31$). In addition, multivariate analysis was carried out with factors of age, gender, performance status, HLA typing, increased peptide-reactive IgG antibodies levels, increased CTL responses, increased cytotoxicity, and observed DTH reaction. Among them, significantly contributed factors for overall survival of 91 cases were increased peptide-reactive IgG antibodies levels ($P = 0.0014$) and performance status 0 or 1 ($P = 0.0046$), although only 4 of 91 cases were performance status 2.

Detailed Analysis of Antibody Responses and Survival Time. To obtain a better understanding of the relationship between antibody response and survival time, representative results of serial measurements of IgG reactive to the peptides for ≤12 vaccinations in the patients whose sera showed the positive responses to the vaccinated peptides were shown in Fig. 2. The result on one peptide per patient was given if a serum reacted to several peptides to save space on the study. The data showed in the other studies that were cited in this one (21–25) or those under submission were not given to avoid double publication. Positive responses were judged by the two criteria. The first criterion is the case if the prevaccination serum showed no reactions, and the postvaccination serum showed at least significant levels of IgG (>0.02 of net OD value at a serum dilution of 1:100) specific to the vaccinated peptides. This case was observed in 50 of 60 patients tested. Some of the cases are shown in Fig. 2. The other criterion is the case if the postvaccination serum showed ≥2-fold increase of the IgG level at a serum dilution of 1:100 than that of the prevaccination serum. This case was observed in the remaining 10 cases. Some of these cases are shown in Fig. 2. There was, however, no apparent difference of the survival time between the two groups (Fig. 3*A*).

In regard to the kinetics, positive antibody responses were induced in sera after the third vaccinations in 19 cases and after the sixth vaccinations in the remaining 41 cases, respectively. There was, however, no apparent difference of the survival time between the two groups (Fig. 3*B*). Among 113 cases shown in

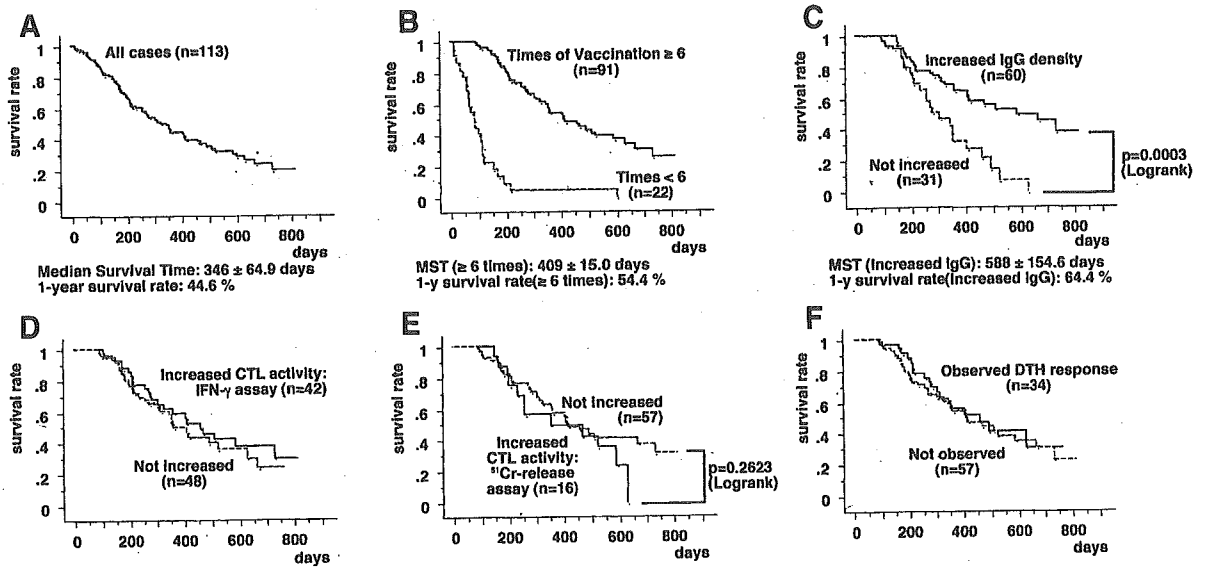

Fig. 1 Overall survival and laboratory markers. In *A*, overall survival of all of the enrolled cases (n = 113) is shown. In *B*, 91 cases received six more vaccinations (one cycle), and 22 cases could not achieve one cycle because of disease progression. In *C*, 60 of the 91 cases, whose postvaccination (third and sixth) sera showed increased levels of peptide-specific IgG, had longer survival (P = 0.0003) than 31 cases, whose sera did not show such increment. *D* and *E*, overall survival curves of cases who had increased CTL activity to the vaccinated peptides (*D*) and cancer cells (*E*). *F*, overall survival curve of cases with observation of delayed type hypersensitivity (*DTH*) response until the sixth vaccination is shown.

this study, a substantial number of cases have been receiving the peptide vaccination for >18 months with stable disease conditions in most cases, and the consistently higher levels are observed in sera of these cases (data not shown).

In regard to numbers of positive peptides, positive antibody responses to only one peptide and at least two peptides were seen in sera of the 35 and 25 cases, respectively. There was also no apparent difference of the survival time between the two groups (Fig. 3*C*). In detail, 35, 18, 6, and 1 showed increased humoral responses to one, two, three, and four peptides among all of the vaccinated peptides (at maximum four), respectively. However, because of too few cases for the analysis, the positive response to more than three peptides was not seen to contribute to overall survival.

In regard to the magnitude of humoral responses, the strong antibody responses by means of increase of IgG levels from the baseline to >0.5 OD values at a serum dilution of 1:100 were seen in of sera 2, 15, 6, and 1 cases after the 3rd, 6th, 9th, and 12th vaccinations, respectively. A part of the results are shown in Fig. 2. The peptides involved in the stronger responses were mainly UBE_{43}, $SART3_{109}$, and lck_{486}.

The 91 cases who received the vaccination for more than six times were divided into the four groups based on their CTL and antibody responses. There was no apparent difference in the overall survival between the cases showing both CTL and antibody responses (n = 30) and those showing only antibody response (n = 30). Similarly, there was no apparent difference in the overall survival between the cases showing only CTL response (n = 12) and those showing neither CTL nor antibody response (n = 19; Fig. 3*D*).

We lastly studied the correlation between antibody response and overall survival in each of the four diseases. Antibody response did not significantly correlate with overall survival in colorectal cancer patients (Fig. 3*E*) or gastric cancer patients (P = 0.6059; data not shown), whereas it well correlated in hormone refractory prostate cancer patients (P = 0.0374; Fig. 3*F*) and lung cancer patients (P = 0.0486; Fig. 3*G*).

DISCUSSION

The detailed results of a Phase I study of CTL precursor-oriented peptide vaccines to each type of cancer have been reported elsewhere (21–25) or are currently under submission for colorectal cancer.[9] In this study, immune responses and clinical benefits were mainly analyzed in all of the patients with various types of cancers under the same regimen to discover a laboratory marker for prediction of prognosis. The results of 91 patients who received more than six vaccinations (one cycle) were used for statistical analysis. Patients (n = 60) whose sera showed increased levels of humoral responses to at least one vaccinated peptide had significantly better prognosis than those whose sera did not show such increased levels. We reported previously that some CTL-directed peptides have the ability to elicit both cellular and humoral immune responses in Phase I clinical studies (20–24). It is well known that humoral response is important for tumor regression. Indeed, we have reported that levels of antipeptide antibodies in postvaccination sera seemed

[9] Y. Sato *et al.*, submitted for publication.

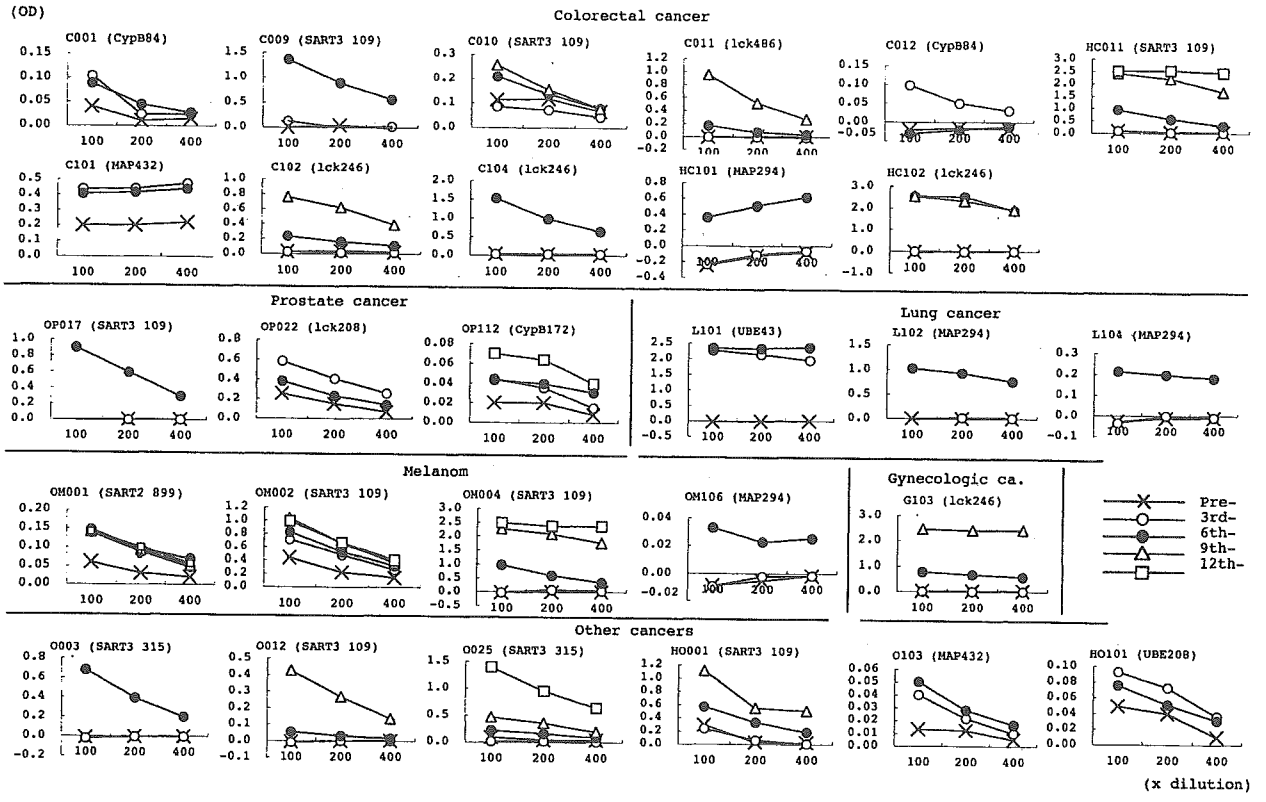

Fig. 2 Measurements of IgG reactive to the vaccinated peptides. Representative results of measurements of IgG reactive to the peptides for ≤12 vaccinations in the patients whose sera showed the positive responses to the vaccinated peptides are shown. It was judged as positive when postvaccination serum showed at least significant levels of IgG (>0.02 of net optimal density (*OD*) value at a serum dilution of 1:100) specific to the vaccinated peptides from a negative level in prevaccination serum. It was also judged as positive when postvaccination serum showed a ≥2-fold increase of the IgG level at a serum dilution of 1:100 than that of the prevaccination serum.

to correlate with the overall survival of advanced lung cancer patients who received peptide vaccination (21). The results shown in this study along with those from the initial results regarding lung cancer suggest that the elevation of humoral response to vaccinated peptides is a favorite factor for patients who received peptide vaccination beyond their different tumor origins. The correlation of humoral responses with better prognosis has also been reported in the other vaccination regimens in which whole tumor cells or epidermal growth factor is used for vaccination in melanoma patients or non-small cell lung cancer patients, respectively (30, 31).

We reported previously that IgG reactive against CTL epitope peptides is often detected in prevaccination sera of cancer patients and also in sera of healthy donors (21–25, 31). We also reported that IgG reactive to these CTL epitope peptides is either lacking or unbalanced in the sera of patients with atopic disease (32, 33). The results shown in this study along with those from noncancerous subjects suggest that these peptide-reactive IgGs play a role in host defense against various diseases, although the underlying mechanism in antitumor immune responses in cancer patients is unclear. These antipeptide IgGs did not react to the mother proteins and also failed to show either the direct inhibition of tumor cell growth *in vitro* or elicit antibody-dependent, cell-mediated cytotoxicity to tumor cells as far as tested (data not shown). Additional studies are needed to clarify their biological role, as well as their mechanism of action.

DTH response is a simple method with high reproducibility and has often been used as a laboratory marker to monitor immune responses *in vivo* for vaccination against infectious diseases and also malignant diseases. However, controversial results have been obtained regarding DTH response as a laboratory marker for either measuring immune responses to antigens or in the prediction of clinical benefits for vaccinated patients (15–28, 30, 31). In this study, DTH response did not correlate with either clinical course or overall survival. In addition, measurements of increased cellular immunity to either peptide or tumor cells did not correlate with overall survival. Collectively, none of the three assays for cellular immunity correlated closely with overall survival, regardless of the fact that the vaccinated peptides were screened by CTL precursor assay in prevaccination PBMCs. There may be several reasons for this unexpected result. One of them could have to do with reproducibility. CTL precursor frequency analysis, enzyme-linked immunospot assay, and cytotoxicity assays are generally used as

Minireview

The role of nuclear Y-box binding protein 1 as a global marker in drug resistance

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Abstract

Gene expression can be regulated by nuclear factors at the transcriptional level. Many such factors regulate *MDR1* gene expression, but what are the sequence elements and transcription factors that control the basal and inducible expression of this gene? The general principles through which transcription factors participate in drug resistance are now beginning to be understood. Here, we review the factors involved in the transcriptional regulation of the *MDR1* gene. In particular, we focus on the transcription factor Y-box binding protein 1 and discuss the possible links between Y-box binding protein 1 expression and drug resistance in cancer, which are mediated by the transmembrane P-glycoprotein or non-P-glycoprotein. [Mol Cancer Ther 2004;3(11):1485–92]

Introduction

Drug export from cells is mediated through a group of proteins belonging to the ATP binding cassette family of transporters. The 170-kDa transmembrane protein P-glycoprotein (PGP), which is encoded by the multidrug resistance 1 (*MDR1*) gene, is a representative example of an ATP binding cassette transporter. PGP consists of two membrane-spanning domains and two nucleotide binding domains and has been reported to affect the pharmacokinetics of drugs by limiting the rate at which they are absorbed (1–5). Various molecules are targeted by drug treatments for cancer; however, PGP expression is responsible for resistance to the widest range of anticancer drugs (6, 7).

The expression of *MDR1*/PGP in human malignant cancers is expected to play a critical role in limiting their sensitivity to anticancer agents. Therefore, the determination of *MDR1* gene expression levels, along with studies of the regulatory mechanisms of this gene, will be useful in developing tailor-made therapeutic strategies for cancer patients.

The partial sequence of the human *MDR1* gene was first reported in the 1980s (8), and its complete sequence, including clustered CpG sites that are not associated with a TATA box, is now known (9). Within the *MDR1* promoter sequence, a GC box forming a Sp1 site and an inverted CCAAT (ATTGG) site for Y-box binding protein 1 (YB-1) or nuclear factor Y (NF-Y) binding both play key roles in *MDR1* gene expression (10).

MDR1 gene expression is often observed in recurrent cancers and appears after the chemotherapeutic treatment of various human malignancies. In cultured human cancer cells, the *MDR1* promoter was activated by both PGP targeting drugs (vincristine and doxorubicin) and non-PGP-targeting drugs (5-fluorouracil and etoposide; ref. 11). In addition, treatment with retinoic acids and other differentiating agents resulted in enhanced expression of the *MDR1* gene product PGP (12). Expression of the *MDR1* gene was also up-regulated by heat shock, arsenate, and serum starvation in cultured human cancer cells (13–16). Consistent with these findings, *MDR1* gene expression was markedly induced by anticancer agents (17); the gene promoter was also activated in response to both anticancer agents and UV light (18, 19). These results show that *MDR1* gene expression is highly susceptible to various environmental stimuli (Table 1) and might therefore be stress responsive (11).

This review focuses on the molecular mechanism of the transcriptional regulation of human *MDR1*/PGP and the role of YB-1 expression in the acquisition of drug resistance.

Transcriptional Regulation of the Human *MDR1* Gene

Many studies have shown the involvement of various *cis*-acting elements in *MDR1* gene expression, suggesting

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Table 1. Transcriptional regulation of the *MDR1* gene in human cell lines

Transcription factor	Inducers	References
NF-Y	None	(40)
	Sodium butylate	(43)
	Trichostatin A	(42)
Sp1	None	(45)
YB-1	UV light	(30, 32)
	Anticancer agents	(31)
Nuclear factor-interleukin-6	Phorbol ester	(52, 78)
EGR1	Phorbol ester	(45, 46)
HSF1	Heat shock	(15, 55)
20-kDa protein	Serum starvation	(16)
Transcription factor 4/β-catenin	None	(56)
Human T-cell lymphotrophic virus-1 Tax	Virus infection	(79)
SXR	Digoxin	(80)
MDR1 promoter-enhancing factor 1/RNA helicase A	None	(59, 60)
Nuclear factor-κB	Daunomycin	(58)
p53	None	(49–51)

pleiotropic mechanisms (10). As shown in Table 1, several transcription factors are expected to play critical roles in the basal expression of the *MDR1* promoter in addition to stimulus-induced activation.

Y-Box Binding Protein 1

Many reports on the factors associated with drug resistance have shown a plausible association of YB-1 with drug resistance both in cultured cancer cells and in numerous clinical human tumor samples.

YB-1 is a member of the cold shock domain (CSD) protein family, which is found in the cytoplasm and nucleus of mammalian cells. It has pleiotropic functions in the regulation of gene transcription and translation, DNA repair, drug resistance, and cellular responses to environmental stimuli (20–22). The structures of YB-1 and two other members of the CSD family, hdbpA (23) and Contrin/hdbpC (24), are presented in Fig. 1A. The *YB-1* gene, which is located on chromosome 1p34 (25, 26), contains eight exons spanning 19 kb of genomic DNA (Fig. 1B). The 1.5-kb mRNA encodes a 43-kDa protein comprising three domains: a variable NH_2-terminal tail domain (A/P domain), a highly conserved nucleic acid binding CSD, and a COOH-terminal tail domain (B/A repeat; refs. 27–29). The A/P domain (amino acids 1-51) seems to be involved in transcriptional regulation, whereas the CSD domain and part of the B/A repeat (amino acids 51-205) function in binding the Y-box (inverted CCAAT box) or double-stranded DNA. Most of the COOH-terminal region of the B/A repeat domain (amino acids 129-324) is thought to bind ssDNA or RNA, and part of this region (amino acids 129-205) is involved in dimerization.

We identified YB-1 as a transcription factor that binds to the inverted CCAAT box of the *MDR1* promoter (30). Decreased expression of YB-1, resulting from the introduction of YB-1 antisense expression constructs into cancer cells, markedly reduced the activation of the *MDR1* gene by DNA-damaging agents (31).

YB-1 is normally present in the cytoplasm, although it is translocated to the nucleus when cells are exposed to anticancer agents, hyperthermia, or UV light irradiation (19, 32, 33). YB-1 is often overexpressed in malignant cells and its expression is regulated by both the proto-oncogene product c-Myc and the tumor suppression gene product p73 (25, 34). The COOH-terminal tail domain seems to play a key role in the localization of YB-1 to either the cytoplasm or the nucleus (32). Studies have shown that cell cycle–specific nuclear translocation is mediated by cooperation of the CSD and COOH-terminal tail domain (35) and that the nuclear translocation of YB-1 requires wild-type p53 (36). The introduction of antisense RNA into human cancer cell lines,[10] and the targeted disruption of one Y-box allele in chicken DT40 cells (37) both inhibited growth. By contrast, the targeted disruption of one allele of the *YB-1* gene in mouse ES-1 cells had no effect on the growth rate (38).

Nuclear Factor Y

The CCAAT box is among the most ubiquitous DNA elements in both forward and reverse orientation. NF-Y is the major transcription factor recognizing the CCAAT box (39). This heteromeric protein is composed of three subunits, NF-YA, NF-YB, and NF-YC, all of which are necessary for DNA binding (Fig. 1A). Mutation and/or deletion of the CCAAT box have been shown to result in a significant loss of *MDR1* promoter activity (40). It has been reported that both the inverted CCAAT box and the GC box are required for activation of the *MDR1* promoter by UV light, and NF-Y, not YB-1, is thought to be the factor regulating the *MDR1* gene (41). However, these findings are not consistent with the results discussed above. The YB-1 protein is abundant and localized in the cytoplasm; however, when the effect of YB-1 overexpression on *MDR1* promoter activity was evaluated in human cancer KB cells, it was unclear whether the nuclear YB-1 content was increased. As YB-1 is known to repress translation, increased levels of cytoplasmic YB-1 might inhibit the translation of luciferase mRNA. Further studies are required to resolve this issue. Treatment with a histone deacetylase inhibitor (trichostatin) induced a marked increase in the amount of *MDR1* mRNA, although this drug-induced increase was inhibited in dominant-negative NF-Y mutants (42). NF-Y therefore seems to regulate *MDR1* gene expression through an interaction with p300/CBP-associated factor, which shows histone acetylation activity. NF-Y might also be responsible for the sodium butyrate–induced *MDR1* gene up-regulation in colon cancer cells (43). This transcription factor therefore plays a pivotal role in *MDR1* gene expression. Recently, the antitumor agent HMN-176, which interacts with NF-YB, has been shown to inhibit *MDR1* gene expression and to restore chemosensitivity to MDR cells (44).

[10] K. Kohno and M. Kuwano, unpublished data.

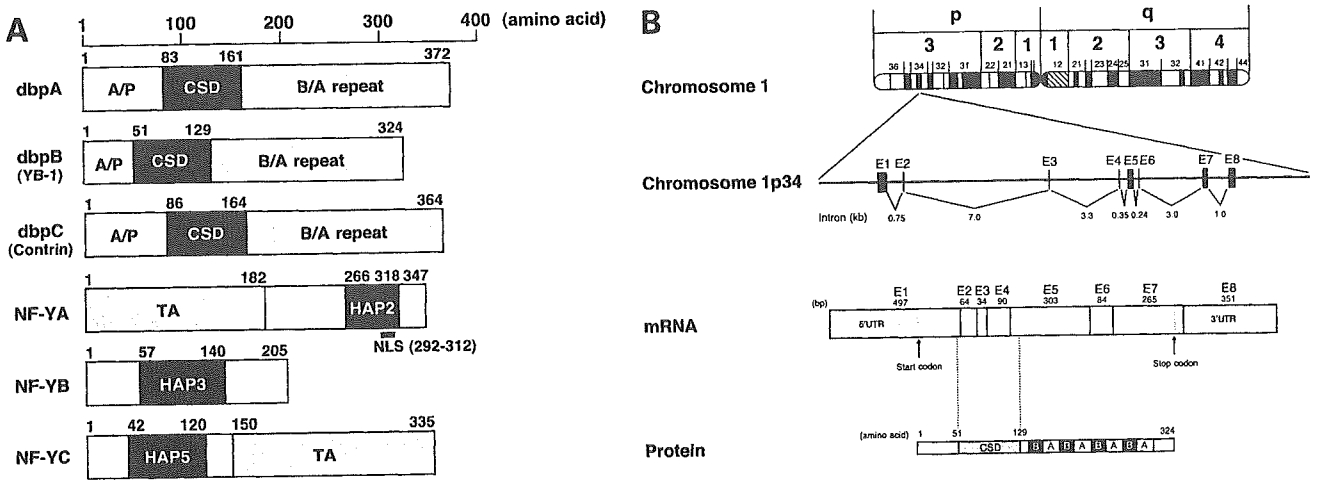

Figure 1. A, protein structure and functional domains of hdbpB/YB-1, hdbpA, hdbpC, NF-YA, NF-YB, and NF-YC. *A/P,* alanine and proline domain, residues 1-82, 1-50, and 1-85 in hdbpA, hdbpB/YB-1, and Contrin/hdbpC, respectively. *CSD,* residues 83-161, 51-129, and 86-164. *B/A repeat,* basic and acidic amino acid, residues 162-372, 130-324, and 165-364. The CSD domains of the three genes are highly homologous. Of the three subunits of NF-Y, NF-YB and NF-YC contain histone folding motifs homologous to the yeast transcription factors *HAP3* and *HAP5*, respectively. NF-YA contains a domain homologous to *HAP2*, which interacts with NF-YB and NF-YC, and the heterotrimer of NF-Y binds to DNA. Both NF-YA and NF-YC contain glutamine-rich domains and activate transcription. **B,** general structure of the genomic DNA, mRNA, and protein product of *YB-1*. The gene is mapped at chromosome 1p34 and has eight exons (*E1, E2, E3, E4, E5, E6, E7,* and *E8*). The YB-1 protein consists of 324 amino acids. *B,* basic amino acid clusters; *A,* acidic amino acid clusters.

Sp1 and Early Growth Response Element 1

The introduction of mutations in the GC-rich region −59 to −45 (G region) of the *MDR1* promoter markedly decreased its activity as a result of the transcription factor Sp1 (40, 45). Sp1 was first cloned and identified as a transcription factor specifically bound to the GC box of the SV40 promoter. A GC box is found in the promoter region of many eukaryotic genes. The Sp1 family is involved in various cellular functions including proliferation, apoptosis, differentiation, and neoplastic changes. As the early growth response element 1 (EGR1) binding motif partially overlaps with the Sp1 binding sites, it is conceivable that they mutually influence *MDR1* gene expression in a competitive manner (45). Treatment with phorbol ester induced the expression of both *EGR1* and *MDR1* genes in human leukemia cells (46). However, the expression of *EGR1* alone did not enhance *MDR1* promoter activity. Coexpression of the oncosuppressor gene *WT1* resulted in the inhibition of *MDR1* promoter activation by EGR1 or phorbol ester (47). Therefore, the direct binding of WT1 to the GC box might compete with Sp1 to down-regulate the *MDR1* gene. These findings suggest that interactions between EGR1 and WT1 might play a key role in *MDR1* promoter activation.

p53

Mutant p53 has been shown to enhance *MDR1* promoter activity in mouse cells; this was reversed by wild-type p53 (14, 48). By contrast, stimulation of the *MDR1* promoter by wild-type, but not mutant, p53 was shown in several human p53-null cancer cell lines. The *MDR1* promoter region −39 to +53 is responsible for this p53-mediated activation (49), whereas the region −189 to +133 is thought to be responsible for negative regulation by wild-type p53 (50). In addition, p53 has been reported recently to bind directly to a novel binding element (−72 to −40) within the *MDR1* core promoter and to repress its promoter activity (51).

Nuclear Factor-Interleukin-6

The treatment of human monocytic cells with phorbol ester enhanced *MDR1* promoter activity through interaction with nuclear factor-interleukin-6, which is a CCAAT/enhancer binding protein family member. This study also revealed that the mitogen-activated protein kinase pathway activates nuclear factor-interleukin-6 (52). In addition, CCAAT/enhancer binding protein β has been shown recently to transactivate the *MDR1* promoter by interaction with the Y-box (53).

Heat Shock Factor

MDR1 promoter activation in response to arsenate or heat shock seems to be mediated through a heat shock element in the −178 to −165 region. An additional region at −136 to −76 has also been proposed as a critical heat shock element for the heat shock response (15, 54), although no direct binding of heat shock factor to this region has been shown. Recently, Vilaboa et al. (55) reported that infection with adenovirus carrying heat shock transcription factor 1 cDNA increased the levels of *MDR1* mRNA and PGP.

Transcription Factor 4/β-Catenin

Transcriptional profiles produced using cDNA microarrays in human colon cancer cell lines identified the *MDR1* gene as the target of transcription factor 4/β-catenin. Seven transcription factor 4/β-catenin binding sites were in the promoter region between −2,030 and +31 (56).

Nuclear Factor-κB

The hepatocarcinogen 2-acetylaminofluorene was shown to activate the *MDR1* gene in human hepatoma cells and the induction of *MDR1* by 2-acetylaminofluorene was mediated by a nuclear factor-κB binding site located around −6 kb (57). Another group showed that the inhibition of nuclear factor-κB reduced levels of *MDR1* mRNA and PGP expression and that nuclear factor-κB transactivated the *MDR1* promoter in human colon cancer HCT15 cells (58). This study identified a nuclear factor-κB binding site in the first intron.

MDR1 Promoter-Enhancing Factor 1/RNA Helicase A

MDR1 promoter-enhancing factor 1 has been shown to bind to the CCAAT sequence causing up-regulation of the *MDR1* gene (59). RNA helicase A has also been reported to bind to the CCAAT box as a member of the *MDR1* promoter-enhancing factor 1 complex (60). Overexpression of RNA helicase A enhanced the expression of both the *MDR1* promoter-reporter construct and endogenous PGP.

Clinical Implications of PGP Expression and Nuclear Translocation of YB-1

PGP triggers resistance to a wide range of anticancer agents including *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, and taxols (7). In addition, YB-1 plays a role in limiting the drug sensitivity of cancer cells by increasing the expression of PGP and other proteins. Immunohistochemical studies of YB-1 expression in the nuclei of untreated primary breast cancers showed an almost complete association between nuclear YB-1 and PGP expression in 9 of 27 cases (Table 2; ref. 61). Studies of clinical specimens have also shown an association between YB-1 and PGP in osteosarcoma (62), synovial sarcoma (63), breast cancer (64, 65), ovarian cancer (66–68), and prostate cancer (Table 2; ref. 69). Figure 2 shows examples of the presence and absence of YB-1 and PGP in clinical samples of osteosarcoma and synovial sarcoma based on the results of immunohistochemical analyses with anti-YB-1 and anti-PGP antibodies.

Table 2. The association of nuclear expression of YB-1 with PGP-mediated and/or non-PGP-mediated drug resistance in human malignancies

Tumor type	Malignant characteristics	References
Ovarian cancer	PGP* ↑	(66)
	PGP* ↑	(67)
	Cisplatin resistance	(68)
Breast cancer	PGP ↑	(61)
	PGP* ↑	(64)
	Drug resistance	(65)
Osteosarcoma	PGP ↑	(62)
Synovial sarcoma	PGP* ↑	(63)
Prostate cancer	PGP* ↑	(69)

*These studies also reported a significant correlation between nuclear YB-1 expression and disease progression or prognosis.

There was a significant correlation between the nuclear expression of YB-1 and the presence of PGP in 69 cases of osteosarcomas (62). A recent study confirmed that YB-1 expression was specifically associated with the overexpression of PGP rather than with three other ATP binding cassette transporters: MRP1, MRP2, and MRP3 (63). By contrast, no association was observed between YB-1 and PGP expression in colon cancers (70). It remains unclear whether YB-1 is directly involved in the transcriptional regulation of PGP in human malignancies. Nevertheless, measurements of the expression of YB-1 and PGP could suggest treatment modalities for individual cancer patients. Recently, we showed that coexpression of YB-1 and PGP correlated with poor prognosis in epithelial ovarian cancer (67). The expression of *MDR1* is augmented in cancerous areas in breast cancer and other tumors, resulting in drug resistance. Furthermore, the presence of YB-1 in the nuclei of cancer cells is closely associated with the clinical outcome. YB-1 could therefore be a useful indicator of malignancy as well as a promising target for cancer therapy. We recommend the use of non-PGP-targeting drugs against malignant tumors with nuclear YB-1 expression.

Clinical Implications of Nuclear Localization of YB-1: Drug Resistance to non-PGP-Targeting Drugs

As described above, YB-1 is translocated to the nucleus in response to various environmental stresses including UV light, anticancer agents, heat, and infection in cultures of cancer cells (21). YB-1 was shown to be overexpressed in cisplatin-resistant cell lines, and antisense YB-1 RNA triggered the augmentation of sensitivity to cisplatin, mitomycin C, UV light, and hydrogen peroxide (30, 38). YB-1 associates with p53 (71) and proliferating cell nuclear antigen (72), both of which modulate DNA repair, cell cycle, transcription, and drug sensitivity. Moreover, wild-type p53 is required for the nuclear translocation of YB-1, which in turn inhibits p53-induced cell death (36). However, it remains unclear how reduced YB-1 expression increases resistance to non-PGP-targeting DNA-damaging agents such as cisplatin and mitomycin C. Potential mechanisms might include a reduction in the YB-1 interaction with proliferating cell nuclear antigen, which is necessary for nucleotide excision repair, or in the interaction with p53. However, pleiotropic drug resistance to DNA-interacting drugs (e.g., aphidicolin, hydroxyurea, cytarabine, etoposide, doxorubicin, and mafosfamide) is associated with the increased expression of YB-1 and 19 other genes that are involved in DNA replication, repair, and stress responses (73).

Nuclear expression of YB-1 was reported to be a prognostic factor in ovarian serous adenocarcinoma (66). It was also associated with cisplatin resistance in ovarian cancer cell lines, and expression levels were increased at some sites of ovarian cancer recurrence (68). This pattern was seen in 7 of 21 serous adenocarcinomas, 2 of 7 clear cell

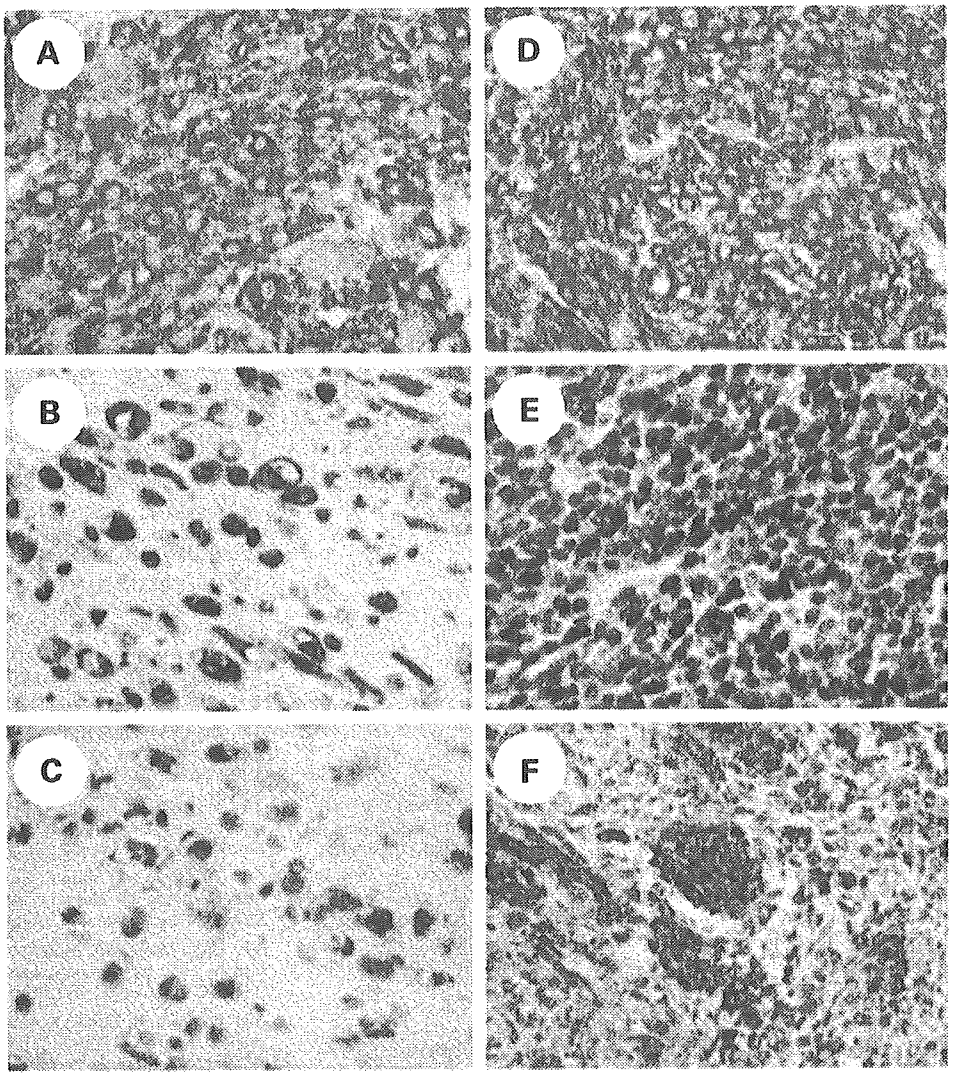

Figure 2. Immunohistochemical detection of nuclear and cytoplasmic YB-1 in osteosarcoma and synovial sarcoma. Antibodies were used against YB-1 (**A**, **B**, **D**, and **E**) or PGP (**C** and **F**). Osteosarcoma is shown with cytoplasmic YB-1 expression (**A**), nuclear YB-1 expression (**B**), and PGP expression (**C**). Synovial sarcoma is shown with cytoplasmic YB-1 expression (**D**), nuclear YB-1 expression (**E**), and PGP expression (**F**). The patient in **D** showed no evidence of disease 131 months after surgery. The patient in **F** died of lung metastasis 8 months after the initial surgery.

adenocarcinomas, and 1 of 4 mucinous adenocarcinomas (Table 2). There was also a positive correlation between the nuclear expression of YB-1 and poor prognosis in synovial sarcoma (63).

Analysis of the clinical relevance of YB-1 expression in the cytoplasm or nucleus in 83 cases of breast cancer, after a median follow-up of 61 months, revealed that the 5-year relapse rate was 66% in patients with high YB-1 expression who received postoperative chemotherapy (65). By contrast, none of the patients with low YB-1 expression experienced relapse. Taken together, these findings indicate that the overexpression and nuclear expression of YB-1 have a predictive value in some human malignancies, both with and without postoperative chemotherapy.

An investigation of 588 genes associated with mouse lung tumor progression revealed that 19 were differentially expressed between lung adenoma and adenocarcinoma; YB-1 was one of these candidate lung tumor progression genes (74). Overexpression of YB-1 was observed in >90% of anaplastic thyroid carcinomas, whereas it was absent in normal follicles and other pathologic tumor types. These findings suggested the involvement of YB-1 in the anaplastic transformation of thyroid carcinoma (75). YB-1 expression induced a strong cellular resistance to malignant transformation through the phosphatidylinositol 3-kinase pathway possibly through the inhibition of protein synthesis that is required for the phosphatidylinositol 3-kinase– or Akt-induced oncogenic transformation (76).

Conclusion

The ancestral protein YB-1 modulates cell growth, apoptosis, drug resistance, DNA repair, transcription, and translation as a pleiotropic regulator. YB-1 overexpression or nuclear YB-1 expression might play a key role not only in the acquirement of PGP-mediated drug resistance but also in sensitivity to non-PGP-targeting chemotherapeutic agents. YB-1 in the nucleus modulates drug resistance to PGP-targeting and non-PGP-targeting drugs in cancer cells

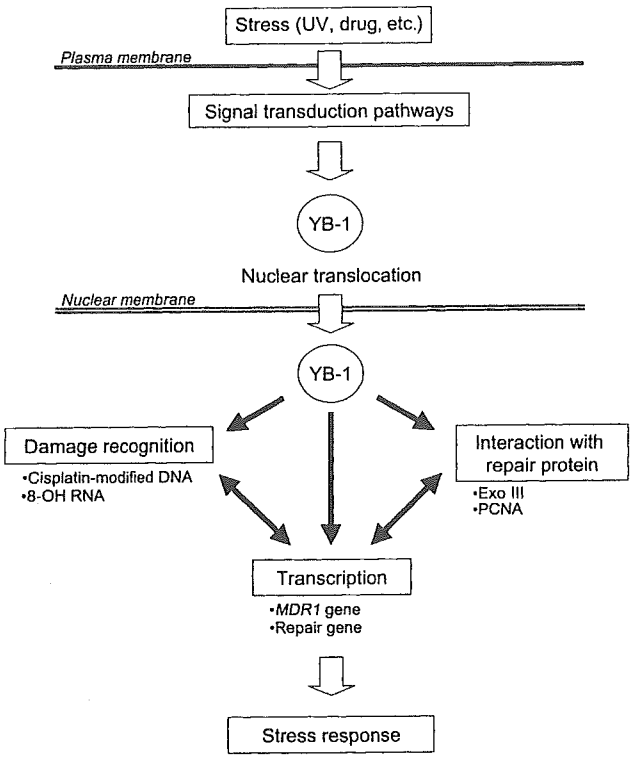

Figure 3. Schematic summary of MDR mediated by PGP or non-PGP. YB-1 is normally present in the cytoplasm but is translocated to the nucleus by treatment with anticancer agents, hyperthermia, or UV light irradiation. YB-1 in the nucleus functions as a transcription factor, which can bind to the Y-box and transactivate promoters, such as the *MDR1* gene or repair genes. By contrast, YB-1 can bind directly to cisplatin-modified DNA and interact with repair proteins including NTH1 (*Exo III*) and proliferating cell nuclear antigen (*PCNA*). These functions might be advantageous for the acquisition of drug resistance.

that are exposed to anticancer and other cytotoxic DNA-damaging agents (Fig. 3). In one response pathway to environmental stimuli, YB-1 is translocated to the nucleus and up-regulates *MDR1* gene expression through binding to the Y-box on the promoter. Alternatively, YB-1 might operate its DNA repair pathway through interactions with p53 (71), proliferating cell nuclear antigen (72), and other molecules (77) when DNA is damaged (Fig. 3). Further research is needed to fully understand the role of YB-1 in cancer and drug resistance.

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