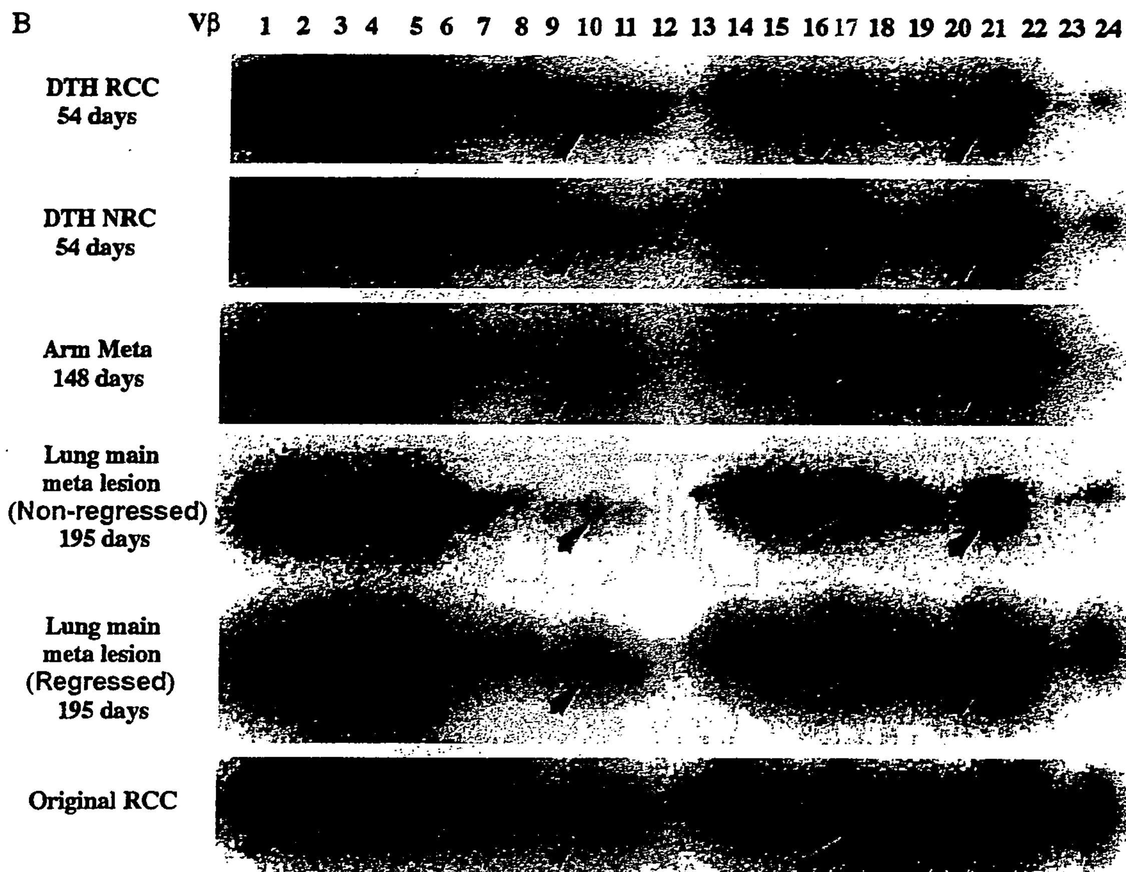

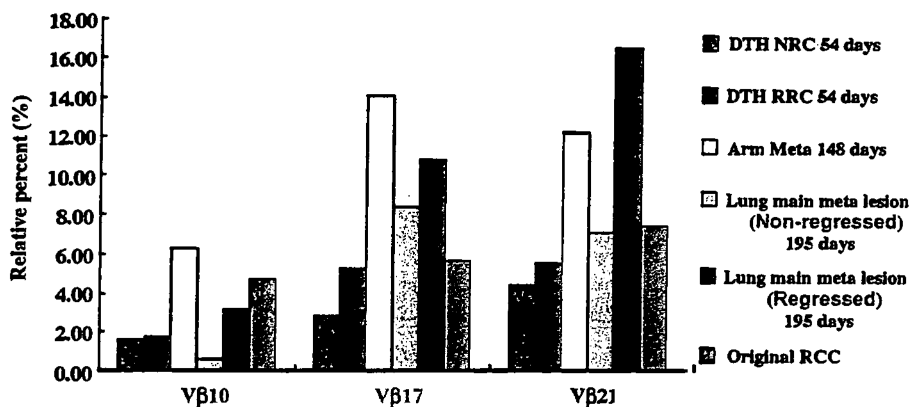

FIG. 5 (*continued*).

adoption of adenovirally transduced GM-CSF autologous tumor cells, allogeneic GM-CSF-transduced cell lines, or autologous tumor cell-based vaccines using GM-CSF-producing bystander cells should be examined as suggested previously [18–20,25–27].

We administered GVAX to four postnephrectomy patients with stage IV RCC without inducing severe vaccine-related adverse events. Furthermore, no remarkable long-term adverse events have been observed in three patients, including two living patients. Our histological findings at the vaccination sites also support the previous observations of triggering the antitumor immune response at these sites [12,14–20,28]. DTH responses in Cases 1, 2, and 3 tended to show stronger reactions to autologous RCC cells than autologous NRC and suggested that anti-RCC-specific immunity was

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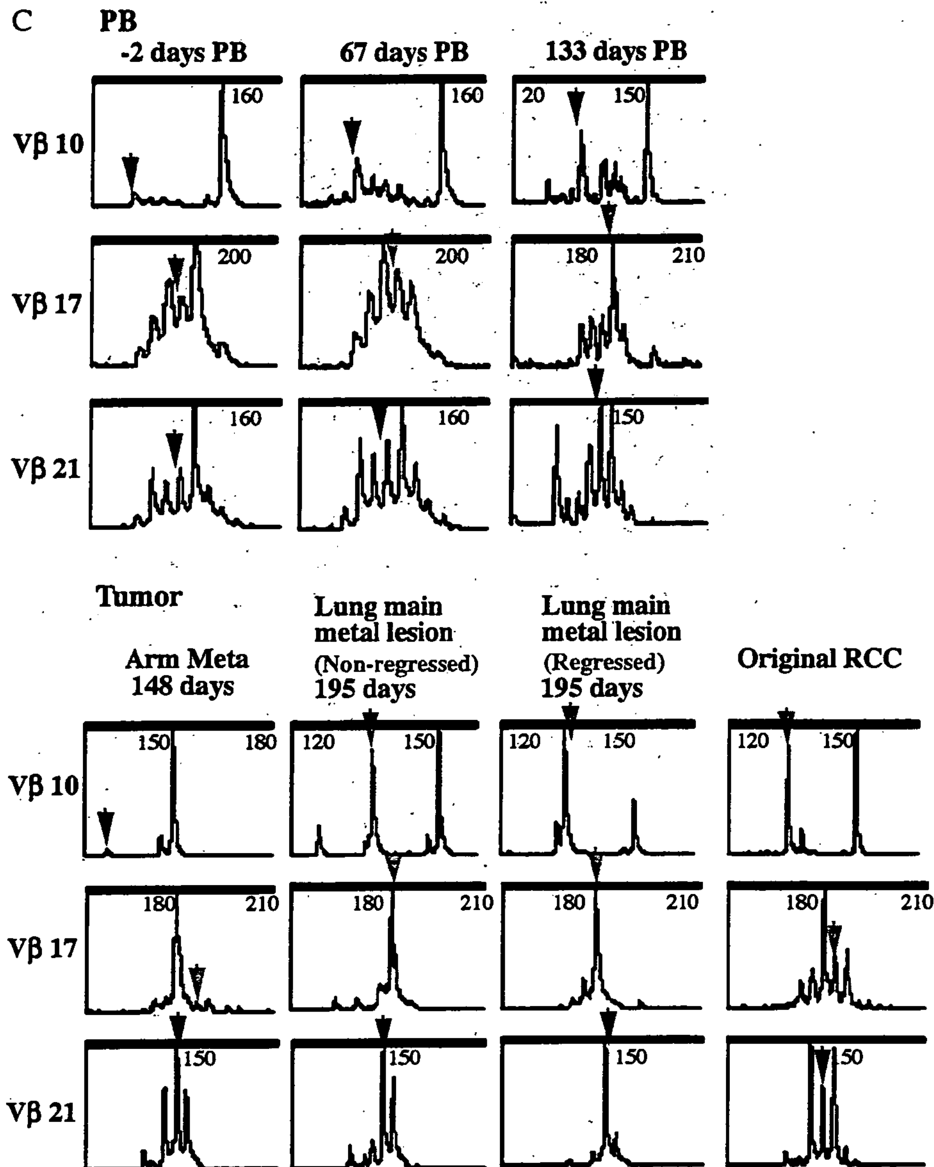

FIG. 5 (*continued*).

induced in these patients. Conversely, the reactions were also positive for NRC, most notably in Case 4, as judged by the skin reaction size (Table 2). The possible causes for this strong background DTH reaction include minimally residual xenoproteins of collagenase or trypsin in GVAX [14] and unknown common antigens existing between NRC and RCC, as the cells that infiltrated into the vaccination sites of RCC and NRC were phenotypically identical, although the number of the former cells was more prominent (data not shown). Thus, possible adverse events of autoimmune nephritis should be monitored carefully. Our follow-up observations over 3 years showed no remarkable renal dysfunction in Cases 2, 3, and 4. Also, Case 1 had no pathological changes associated with autoimmune nephritis in autopsy specimens 7 months after the vaccinations (data not shown). This long-term observation might support the safety of GVAX and its capability of inducing anti-RCC-specific immunity.

The significance of our effector-phase pathological analysis should be emphasized. We had a chance to

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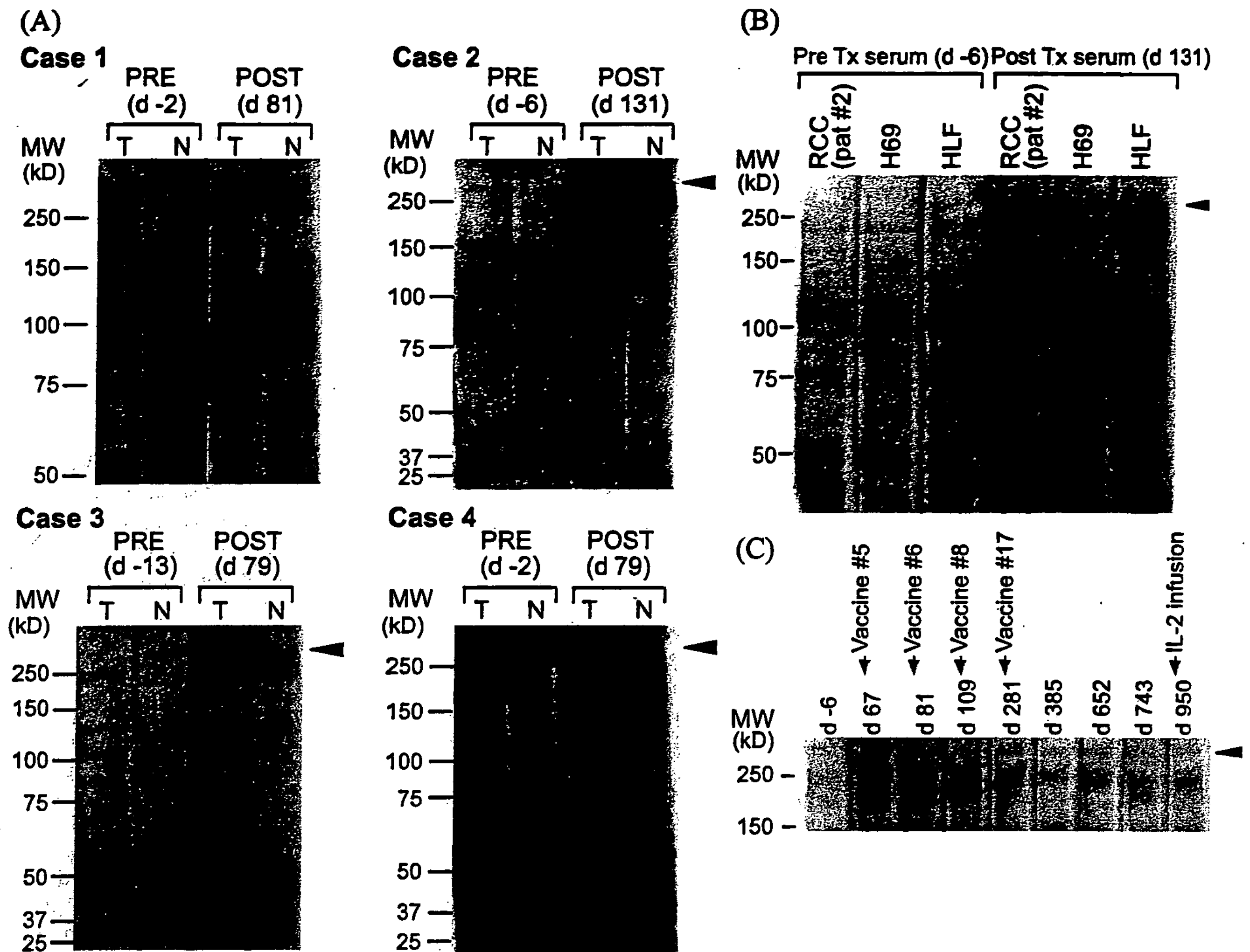

FIG. 6. Appearance of antitumor antibody responses in Cases 1 to 4, who received GVAX. (A) Comparisons of serum reactivity to autologous RCC proteins pre-GTx and post-GTx. Proteins extracted from cultured tumor cells (lane T) or normal kidney cells (lane N) were electrophoresed, transferred onto PVDF membranes, and detected with autologous patient sera (Cases 1 to 4). Sera were harvested before (PRE) and after (POST) the first vaccination (day 0). (B) Comparison of the serum reactivity to autologous RCC, H69 cells, and human lip fibroblasts (HLF). The reactivity to high-molecular-weight proteins was significant in autologous RCC and HLF, while it was weak in H69 cells. Autologous RCC from Case 2 (pat #2) was used. (C) Time course of changes in serum reactivity to high-molecular-weight proteins of approximately 250 kDa in Case 2. Immunoblotting of RCC proteins from Case 2 with autologous sera harvested at several postvaccination days (post-5th, 6th, 8th, and 17th vaccination and before the administration of low-dose IL-2).

investigate pathologically the RCC before and after vaccinations in Case 1 and have demonstrated the induction of tumor site-specific infiltration of predominantly $CD8^+$ T cells. This was associated with tumor apoptosis in postvaccinated biopsy and autopsy tumor specimens, whereas $CD4^+$ T cells predominated and tumor cell apoptosis was negligible in the original RCC (Fig. 3). Notably, these changes were demonstrated in the biopsy specimen that was obtained before IL-2 administration. These observations strongly suggest the induction of tumor-specific immunity by GVAX. Although GVAX could induce both the localization of $CD8^+$ cells within metastatic tumors and significant apoptosis, not all of the tumors showed regression. Thus, GVAX-induced antitumor immunity per se may not be sufficient for clinical efficacy.

We studied various parameters, as it is still unknown which immune factors can be used to predict the therapeutic efficacy of antitumor immune gene therapy. The results of our *in vitro* assessment of cytokine production were compatible to those reported by Soiffer *et al.* [13]. These cytokine profiles indicated the coordinate expression of gene products associated with both Th1 and Th2 cells and suggested that multiple lymphocyte effector mechanisms contribute to the potent antitumor immune response. The cytokines produced by

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these CD4+ T cells activate eosinophils, as well as macrophages that produce both superoxide and nitric oxide. Both of these cell types then collaborate at the site of the tumor challenge to cause its destruction [13]. Our observation suggested that this Th2-dominant immunological response was particularly enhanced, namely, an *in vivo* immune shift from Th1 dominance to Th2 dominance was induced after repeated vaccinations and maintained. Previous studies of GM-CSF immune gene therapy assayed cytotoxicity using PBMC or TIL [13–15]. Kusumoto *et al.* reported that vaccination with irradiated autologous GM-CSF-producing melanoma cells appeared to increase the cytotoxicity against autologous tumor cells in five patients, although repeated vaccination appeared to decrease the CTL activity in two of these cases. They suggested that vaccination of these patients with autologous melanoma cells caused T cell anergy or tolerance [14] without demonstrating the precise underlying immune mechanism involved. In the present study, Case 1, with large lung metastases, which might have contributed to the observed immunological suppression, showed similar results. Although cytotoxicity assayed using PBMC gradually decreased in Case 1, our pathological findings in the metastatic lesion showed the predominant infiltration by CD8 T cells. These findings might support the limited predictability of the *in vivo* antitumor reaction using only traditional immunoassays using PBMC.

Recently, T cell receptor β chain repertoire analysis methods were reported to facilitate the detection of clonal T cell expansion in various biological specimens. As RCC is thought to be a tumor whose growth may be controlled by the immune response, characterization of T lymphocytes found in RCC patients may demonstrate this important issue [16,17]. Using CDR3 length pattern analysis, Puisseux *et al.* demonstrated a selective localization of oligoclonal T cell populations in malignant tissues after comparisons to the T cell repertoire in the tumor and in the autologous peripheral blood lymphocytes or normal adjacent kidney [16]. Importantly, in our clinical studies, the induction of oligoclonal expansion of T cells with the selected TCR in the peripheral blood, skin biopsy specimens from DTH sites, and tumors was demonstrated after vaccination. The reasons for the observed different clonal T cell expansions in the different tissues in our studies may arise from either a polymorphic T cell response to the same antigen or a different immunogenic environment [17]. Hanada *et al.* recently demonstrated the important role of posttranslational protein splicing in the immune recognition of self and foreign peptides using human RCC antigens, and this phenomenon may explain our results [29]. Although we could not prove directly that these oligoclonally expanded T cells responded to RCC antigens, our findings of the generation of MHC-restricted and TCR-mediated cytotoxicity against autologous RCC and the predominant infiltration of CD8 T cells and apoptosis in metastatic lesions [1] supported this possibility.

In addition to the enhanced antitumor cellular immunity, GVAX is thought to induce antitumor humoral immunity. Simons *et al.* measured increased titers of antibodies recognizing prostate tumor antigens in sera from patients vaccinated with GM-CSF-transduced autologous prostate tumor cells. New antibodies recognizing polypeptides of 26, 31, and 150 kDa in extracts from LN CapPCA cells were observed in three of eight patients following the final vaccinations [18]. Soiffer *et al.* reported similar observations, with antibodies recognizing different polypeptides, in melanoma patients [13]. In the present study, Western blot analysis identified RCC-derived polypeptides of 65 and 250 kDa. We are currently screening RCC cDNA expression libraries with our patients' sera using the SEREX method to look for RCC-specific antigens other than RAGE and G250 [30,31]. We have already cloned several candidate cDNAs and are studying their RCC specificities and the possibility of their future application in anti-RCC immunotherapy.

Currently, several candidate strategies to enhance the systemic anti-RCC immunity of GVAX can be considered. These include the coadministration of IL-2 to enhance basal antitumor immunity [3,5–7,32–34], allogeneic stem cell transplantation including nonmyeloablative stem cell transplantation to introduce allogeneic immunity [27], IL-12 or CD80 cDNA-transduced autologous tumor cells for the direct activation of CTLs, the blockade of CTLA-4/B7 interactions with monoclonal antibody to activate costimulation signals, and the functional activation of dendritic cells using HSP gp91 [35–38]. The administration of low-dose IL-2 as an anticancer immunotherapy has recently been introduced to decrease both the side effects and the cost of treatment [39–42].Our experience with three patients who were given GVAX followed by low-dose IL-2 would cast new light on anti-cancer immunotherapy, possibly by inducing tumor-specific immunity by GVAX, followed by enhancement of the broad antitumor immunity with systemic low-dose IL-2. In the present study, *in vitro* CTL analysis in these two patients supported the hypothesis that the antitumor CTL activity was maintained after administration of IL-2. The optimal duration of treatment with low-dose IL-2 in combination with GVAX remains to be determined by closely monitoring antitumor immunity both *in vitro* and *in vivo*.

PATIENTS AND METHODS

Selection of Patients

The details of the study design and methods of vaccine production were essentially the same as those reported by Simons *et al.* [13,14], except for modifications that were implemented according to the regulations for

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clinical gene therapy announced by the Japanese government between 1995 and 1997. Briefly, patients with stage IV RCC (Union Internationale Contre le Cancer classification of 1997) were eligible. Chemotherapy, radiotherapy, systemic IL-2- or interferon-α-based regimens, or other investigational agents were also offered as treatment options to these patients. The following eligibility criteria were used: primary RCC in place with evaluable metastasis after nephrectomy; Eastern Cooperative Oncology Group performance status of zero or one; appropriate surgical candidate and estimated life expectancy of at least 6 months; no major surgery, radiotherapy, chemotherapy, immunotherapy, or immunosuppressive medications within 1 month prior to enrollment; age >18 years; absence of active infection, i.e., WBC count <4000/μl, platelets <100,000/μl, total bilirubin <1.5 mg/dl, and creatinine <2.0 mg/dl; HIV seronegativity; and no history of autoimmune disease. The exclusion criteria included age <20 years; pregnant or lactating women; double malignant tumors; surgery; local or systemic treatment with corticosteroids; immunotherapy; irradiation or anti-cancer drugs 1 month before registration; leukocytosis of unknown origin; history of systemic lupus erythematosis, sarcoidosis, rheumatoid arthritis, autoimmune hemolytic anemia, autoimmune thyroiditis, glomerulonephritis, or vasculitis; apparent infection requiring treatment before second stage; apparent brain metastasis detected on CT scan or MRI; postnephrectomy deep vein thrombosis or pulmonary embolism that required treatment; and opium or alcohol abuse. The study was reviewed and approved by the Committee on Clinical Investigation and Institutional Gene Therapy Ethical Committee, The Institute of Medical Science, University of Tokyo, in April 1998, and by the Joint Committee of the BioScience Committees of the Ministry of Health, Labor, and Welfare and the Ministry of Education, Culture, Sports, Science, and Technology in August 1998.

Study Design

Patients were enrolled from September 1998 to May 2001. Eligible patients were nephrectomized after giving their initial informed consent. The second informed consent was obtained after safety confirmation tests, which included negative tests for microbial contaminants such as bacteria, fungi, mycoplasma, RCR, and endotoxin, and when sufficient production (>40 ng/10^6 cells/24 h) of GM-CSF was detected in the GM-CSF gene-transduced RCC. The vaccination schedule of GVAX, including additional vaccinations, is described precisely under Vaccine preparation and administration. Peripheral blood was obtained (as per NIH Recombinant DNA Advisory Committee and Food and Drug Administration guidelines) for detecting RCR before treatment, after vaccination, monthly for 3 months, every 3 months for the next 9months, and then yearly [14]. Long-term follow-up, including periodic evaluation for autoimmune disease and tumor progression, was performed.

Clinical Evaluation

The patients received daily physical examinations and periodic laboratory tests, which included hematological parameters and liver, renal, and immunological functions, prior to and after the vaccinations. The metastatic lesion volumes were measured using CT (lung, liver, bone, brain), MRI (liver, bone, brain), and thallium or technetium scintigraphy (whole body). Unenhanced helical CT images that covered each lesion were obtained during a single breath-hold. The thickness of the slices ranged from 3 to 10 mm, depending on the lesion size. The data were transferred to a workstation (Advantage Windows; General Electric Medical Systems, Milwaukee, WI, USA) to calculate the tumor volumes. Low-density areas, which represent lung parenchyma, were excluded at a threshold of −400 HU, and lesion sections were selected manually from the remaining areas of each slice. The lesion volume was calculated with a 3-D utility on the workstation for Cases 1, 2, and 3. The lesion in Case 4 was calculated as the sum of the perpendicular diameters of all lesions measured by CT scan, due to difficulties in measuring small multiple tumors volumetrically.

Vaccine Preparation and Administration

The methods used for autologous RCC vaccine preparation and MFGS-GM-CSF gene transfer at the Clinical Cell Processing Facility of the Institute of Medical Science Hospital at the University of Tokyo have been described previously [13]. The procedure complied with good manufacturing practices. Primary cultures were established and transduced at the first passage. Following *in vitro* expansion, the vaccine cells were irradiated at 150 Gy to prevent clonogenic survival *in vivo* after vaccination. GM-CSF production was determined using a GM-CSF ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Genomic integration of the GM-CSF cDNA into the patients' autologous RCC cells was determined by the standard Southern blotting method using MFG-GM-CSF plasmid DNA to determine the copy number, as described elsewhere [43]. The tests for microbial contaminants, i.e., bacteria, fungi, mycoplasma, RCR, and endotoxin, were all performed by BioReliance Corp. (Rockville, MD, USA). The vaccine cells were stored in liquid nitrogen until use. On the day of vaccination, 4×10^7 viable cells were administered intradermally in the first injection, and thereafter, 2×10^7 cells were administered at least five times at 2-week intervals, which was considered to be a superior vaccination schedule as described by Soiffer *et al.* [16]. Each patient was carefully screened for eligibility according to the inclusion criteria by the Institutional Review Board (IRB) of the Institute of Medical Science, University of Tokyo. The IRB permitted additional

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administration of vaccine every 2 weeks when the yield of cells was higher than the 1.4×10^8 cells required for the six scheduled administrations and in cases in which the patient's physical condition was acceptable after further informed consent was obtained. The vaccinated sites were biopsied for microscopic examination at 3 and/or 7 days after every second vaccination.

Toxicity Assessment and Pharmacokinetic Analysis of Serum GM-CSF Levels

The levels of toxicity were graded using the National Cancer Institute's cancer common toxicity criteria for clinical trials. Toxicities were identified by medical history, physical examination, and review of the laboratory studies performed. Patients' sera were frozen in 1-ml aliquots at −80°C until the day of testing. The serum GM-CSF levels were determined for all collection time points by enzyme-linked immunosorbent assay using the Biotrak human GM-CSF ELISA system (Amersham International Plc., Amersham, UK) according to the manufacturer's protocol.

Histological Studies

Six-millimeter punch biopsies were removed from the intradermal injection sites on days 3 and/or 7 following the first vaccination. Prevaccination skin biopsies were obtained for comparison. Similarly, skin biopsies were also taken for evaluation of the DTH reaction 48 h after intradermal inoculation of RCC cells and NRC. Surgically removed and autopsy materials were used for the histological evaluation of tumors and tumor-infiltrating cells. Biopsy materials were fixed in 10% buffered formalin, embedded in paraffin, stained with H&E, and labeled with antibodies to CD3, BMP (rabbit antiserum to human myelin basic protein; DAKO Corp., Carpinteria, CA, USA), AE1/AE3 (pooled mAbs to human epithelial keratin, IgG1 subtype; Boehringer Mannheim, Indianapolis, IN, USA), S100 (rabbit anti-cow S100; DAKO), CD68 (anti-human macrophage CD68 mAb, IgG3-subtype; DAKO), HLA-DR (clone LN3, IgG2a subtype; Lab Vision Corp., Fremont, CA, USA), CD3 (clone PS1, mAb, IgG2a subtype; Novocastra Laboratories, Newcastle, UK), CD4 (clone 1F6, mAb, IgG1 subtype; Novocastra Laboratories), CD8 (clone 1A5, IgG1 subtype; Novocastra Laboratories), and CD20cy (B cell marker, clone L26, mAb, IgG2a subtype; Lab Vision Corp.). For the evaluation of tumor apoptosis, the TdT-mediated dUTP–biotin nick end-labeling (TUNEL) method was applied using an ApopTag Kit (Intergen Co., Purchase, NY, USA).

Delayed-Type Hypersensitivity Testing

To evaluate the cell-mediated immunity status of each patient before and after treatment, DTH testing was performed using seven common recall antigens (Multitest CMI; Connaught Laboratories, Swiftwater, PA, USA) according to the manufacturer's instructions. Reaction scoring was also performed according to the manufacturer's instructions. The patients were tested simultaneously for reactivity to autologous, irradiated cultured RCC cells and NRC. The autologous RCC cells and NRC for DTH testing were prepared and stored in liquid nitrogen according to the same procedure used for vaccine cell production omitting GM-CSF transduction. During storage, sterility testing for bacteria, fungi, mycoplasma, and endotoxin was carried out at the Department of Laboratory Medicine, Institute of Medical Science, University of Tokyo. PBMC were isolated using the standard Lymphoprep (d = 1.077; Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation method. These cells were washed three times with HBSS, counted, and injected intradermally at 10^6 cells/0.2 ml. DTH reactions were observed 48 h after each DTH injection, i.e., 1 week before the first vaccination and 1 week after the second, fourth, and sixth vaccinations in all four patients.

Tumor Tissues, Peripheral Blood, and Skin Biopsies from Patients

Single-cell suspensions of tumor tissues were obtained from biopsied or autopsied (Case 1) tumor specimens that were minced mechanically and treated with collagenase and DNase. RCC cells and TILs were separated by density gradient centrifugation, as described elsewhere [24]. Heparinized peripheral blood samples (20 ml) were drawn from patients every other week before vaccination. For follow-up, samples were also drawn when the patients permitted. Patients' sera were frozen at −80°C until use for Western blot analysis. PBMC were isolated as above. PBMC and TIL (5×10^6 cells/tube) were cryopreserved using a programmable freezer and stored in liquid nitrogen. In addition, the cell pellets were frozen in liquid nitrogen until used for RNA extraction. Skin biopsies obtained from the DTH reaction site (6 mm in diameter) were cut into pieces measuring approximately 1 × 1 mm and rapidly frozen in liquid nitrogen until used for RNA extraction.

Assessment of Lymphocyte Proliferation and Cytokine Production

On the day of the assay, the cryopreserved samples were thawed. PBMC (1×10^5 cells/well) were cultured in the presence of irradiated (150 Gy) GM-CSF-transduced autologous tumor cells (1×10^4 cells/well) plus IL-2 (40 U/ml), in 96-well flat-bottomed plates. RPMI 1640 medium with L-glutamine (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA) and gentamicin was used as complete medium. On day 3 or 6, culture supernatants (100 µl/well) were collected from each well to determine the cytokine levels, and fresh medium was added. The cultures were then pulsed with [^{3}H]thymidine (0.5 µCi/well; DuPont-NEN, Boston, MA, USA) for a final 18 h and harvested on a Micro 96 harvester (Skatron, Lier, Norway), and the incorporated radioactivity was measured using a

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microplate counter (Micro Beta Plus; Wallac, Turku, Finland). ELISAs for human IFN-γ, IL-5, and IL-10 were performed using ELISA kits (OptEIA; BD-Biosciences, Boston, MA, USA) according to the manufacturer's protocols.

Cytotoxicity Assay

To prepare effector cells, cultures with IL-2 and irradiated GM-CSF-transduced autologous tumor cells as described above were prepared in 96-well round-bottomed plates and the plates were cultured for 7 days. On the day of the assay, aliquots of 100 μl of the culture medium were removed from each well and then labeled target cells (5×10^3 cells/100 μl/well) were added. To label the target cells, single-cell suspensions of cultured autologous or allogeneic RCC cells, autologous NRC, and K562 cells were incubated with $Na_2^{51}CrO_4$ (100 μCi) for 1 h at 37°C and washed three times prior to use. For blocking experiments, F(ab′)2 anti-CD3 mAb prepared as described previously [44] was added to a final concentration of 10 μg/ml at the start of the assay. The plates were incubated at 37°C for 6 h, the supernatants were collected using a Skatron cell harvester system (Diversified Equipment Co., Lorton, VA, USA), and the radioactivity was measured using a γ counter. Spontaneous release (SR) and maximal release (MR) were measured in the supernatant of target cells alone with 100 μl of either medium or 10% Triton X-100 (Sigma, St. Louis, MO, USA). The percentage specific cytotoxicity was calculated using the following formula: % cytotoxicity = experimental release − SR/MR − SR × 100.

Analysis of the TCR β Repertoire

Total RNA was isolated from PBMC and homogenized tumor tissues using Trizol reagent (Invitrogen) with a cryo-press crusher (Microtech Nichion, Tokyo, Japan). TCR β repertoire analysis was performed as described previously [45]. Briefly, TCR β cDNA was synthesized using C-oligonucleotides (5′-CGGGCTGCTCCTT GAGGGGCTGCG-3′) with AMV reverse transcriptase (Invitrogen). The TCR cDNA was amplified by 40 cycles of PCR with each of the 24 Vβ 5′ primers (Vβ 1-w24) and the Cβ 3′ primer in PCR buffer containing 1 U of Hot Start *Taq* polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, CA, USA). The products were subjected to Southern blot analysis using a ^{32}P-labeled Cβ probe. Different samples of each Vβ product were compared after quantifying the autoradiographs by densitometry BAS-2000II (Fuji Photo Film Corp.). To refine CDR3 size analysis, the Vβ–Cβ PCR product was copied in a 10-cycle run-off reaction with a fluorescence-labeled Cβ primer. The labeled PCR products were electrophoresed on a DNA sequencer (ABI Prism 377; Applied Biosystems) in the presence of a fluorescent size standard and analyzed with a DNA fragment size program (GeneScan; Applied Biosystems).

The PCR products of the CDR3 fragment were cloned into the pCRII-TOPO vector system (Invitrogen). Thirty colonies containing the insert fragment were selected at random and sequenced using an ABI Prism Cycle Sequencing Kit (Applied Biosystems) and an automatic DNA sequencer ABI 373 (Applied Biosystems). The amino acid sequence of the CDR3 region was deduced using the software GENETYX-MAC v10.1.4 (Software Development Co., Ltd., Tokyo, Japan).

Detection of Antitumor Antibodies

The antitumor antibodies appearing in patients' sera were detected by Western blot analysis according to the standard procedure with some modifications [18]. Briefly, humoral antitumor immune responses were evaluated using the reactivity of the tumor cell lysate and sera from the patients. Autologous RCC and NRC were extracted in lysis buffer containing 20 mM Tris–HCl at pH 7.6, 1% NP-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 500 units/ml aprotinin (Calbiochem, La Jolla, CA, USA). A fibroblast cell line of human lip origin, which was established in our laboratory, and a small-cell lung carcinoma cell line, H69, were used as irrelevant control cells. Cell lysates were denatured, reduced in SDS sample buffer with 2-mercaptoethanol, and then electrophoresed on 7.5% polyacrylamide minigels (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were transferred onto Immobilon membranes (Millipore, Bedford, MA, USA) and the blots were stained with Ponceau S solution (Sigma) for visualization. After destaining with TBST (0.1% Tween 20–Tris-buffered saline) and blocking with 5% nonfat dried milk in TBST overnight, the blots were probed with diluted (1:300) patient sera for 2 h. Horseradish peroxidase-conjugated rabbit F(ab′)2 anti-IgG Ab (DAKO, 1:3000 dilution) was added for 1 h, and the blots were developed with an ECL kit (Amersham Biosciences, Piscataway, NJ, USA).

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Effectiveness of Adjuvant Intermittent Endocrine Therapy Following Neoadjuvant Endocrine Therapy and External Beam Radiation Therapy in Men With Locally Advanced Prostate Cancer

Hidetoshi Yamanaka,[1] Kazuto Ito,[2*] Seiji Naito,[3] Taiji Tsukamoto,[4] Michiyuki Usami,[5] Hiroyuki Fujimoto,[6] Naoki Matsuoka,[6] Iwao Fukui,[7] Masaoki Harada,[8] Yasuo Ohashi,[9] Toshihiko Kotake,[10] and Tadao Kakizoe[6]

[1]Institute for Preventive Medicine, Kurosawa Hospital, Takasaki, Japan
[2]Department of Urology, Gunma University Graduate School of Medicine, Maebashi, Japan
[3]Department of Urology, Graduate School of Medicine, Kyusyu University, Fukuoka, Japan
[4]Department of Urology, Sapporo Medical University School of Medicine, Sapporo, Japan
[5]Department of Urology, Osaka Medical Center for Cancer and Cardiovascular Disease, Osaka, Japan
[6]Urology Division, National Cancer Center Hospital, Tokyo, Japan
[7]Department of Urology, Cancer Institute Hospital, Tokyo, Japan
[8]Kanagawa Cancer Center Research Institute, Yokohama, Japan
[9]Department of Hygiene and Preventive Medicine, School of Health Sciences and Nursing, University of Tokyo, Tokyo, Japan
[10]Kotake Clinic, Osaka, Japan

PURPOSE. To clarify the optimal duration and methods for adjuvant endocrine therapy after external beam radiation therapy (EBRT) in patients with locally advanced prostate cancer.
MATERIALS AND METHODS. Between 2001 and 2003, 215 patients with locally advanced prostate cancer were enrolled in the study. Patients were registered as primary candidates of the study and were treated with 6 months of LHRH agonist, with short-term of antiandrogen treatment for flare-up prevention. Patients with PSA levels below 10 ng/ml after the 6-month endocrine treatment were randomly divided into two arms. Then, a total dose of 72 Gy was given to the prostate. After 14 months of the protocol treatment, patients were treated with continuous androgen ablation (arm 1) or intermittent androgen ablation (arm 2).
RESULTS. A total of 188 cases (87%) remained in the protocol. The median PSA level at entry was 25.3 ng/ml. The Gleason score was 2–6 in 32 cases (16%), 7 in 94 cases (48%), and 8–10 in 68 cases (35%). The median PSA level showed a remarkable decrease to 1.1, 0.2, and 0.1 ng/ml, after 6, 8, and 14 months of the protocol treatment, respectively. Of the 157 cases treated with EBRT, 153 cases (97.5%) had no biochemical failure in the mean follow-up of 17.3 months.

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*Correspondence to: Kazuto Ito, MD, Department of Urology, Gunma University Graduate School of Medicine, 3-39-22, Showa-machi, Maebashi, Gunma, 371-8511, Japan.
E-mail: kzito@med.gunma-u.ac.jp
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CONCLUSIONS. The present study may reveal the possibilities of intermittent endocrine therapy after EBRT. However, the follow-up interval is short and little can be said about the results observed so far, exception of acute tolerance and patient acceptance of the protocol.
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KEY WORDS: **prostate cancer; intermittent hormonal therapy; external beam radiation therapy**

INTRODUCTION

Treatment of prostate cancer has been one of the most important issues for elderly males, especially in Western countries. In Japan, prostate cancer is the eighth leading life-threatening cancer in males [1]. However, in the past 10 years, the probability of cause of death from prostate cancer has increased and will increase rapidly in the future [1,2]. In the present study, we have conducted a prospective randomized control trial (RCT) for locally advanced prostate cancer in order to clarify how to treat it with adjuvant endocrine therapy after external beam radiation therapy (EBRT). The previous RCT for locally advanced prostate cancer already revealed that cancer causes of death and also all causes of death may decrease in men treated with both EBRT and endocrine therapy (neoadjuvant and/or adjuvant) in comparison with those treated with EBRT alone [3–5]. Bolla et al. [3] demonstrated that 5-year disease-free survival was higher at 85% in patients with locally advanced prostate cancer treated with EBRT and 3 years of endocrine therapy than in those treated with EBRT alone. However, the optimal timing and duration for endocrine therapy as adjuvant or neoadjuvant treatment with EBRT have not been solved. Furthermore, those issues should be discussed in terms of not only survival advantage, but also improvement of QOL.

Alternatively, the concept of intermittent endocrine therapy was proposed as a possible treatment to prolong the hormone naïve status of prostate cancer. According to basic research on androgen-dependent Shionogi carcinoma in mice, androgen-dependent status recovered after endocrine treatment was stopped in hormone-independent prostate cancer. This phenomenon would result in induction of apoptosis several times during intermittent androgen deprivation [6]. Although the treatment efficacy of intermittent hormonal therapy has not been confirmed in clinical settings, there may be some advantages in the cost for treatment, prevention of osteoporosis development, and recovery of libido.

The present assessment of combination therapy with EBRT and endocrine therapy for locally advanced prostate cancer may be of positive concern. However, it may be difficult to answer how long neoadjuvant and/or adjuvant endocrine therapy should be used. Several RCTs have been carried out or are ongoing in Europe and the USA. However, there have been no RCTs comparing the treatment efficacy and QOL between long-term adjuvant endocrine therapy and intermittent adjuvant endocrine therapy after treatment with EBRT and neoadjuvant endocrine therapy for locally advanced prostate cancer. To answer uncertainties on the above issues, the present multi-center RCT was conducted as a national cancer research project, which has been supported by the Ministry of Health, Labor and Welfare in Japan.

The primary endpoint of this study is biochemical relapse-free survival and the secondary endpoints are overall survival, cancer-specific survival and longitudinal QOL assessment between two groups. It is expected that the survival advantage by means of biochemical relapse-free survival in the continuous adjuvant endocrine treatment group may be better than that in the intermittent endocrine treatment group. Alternatively, adverse effects in patients treated with long-term androgen deprivation may increase in comparison with those treated with intermittent androgen deprivation. After completing this RCT, we expect to be able to distinguish patients who can benefit more from continuous hormonal treatment by means of survival with minimized adverse effect from those who can benefit more from intermittent hormonal treatment by means of maintaining QOL without dying of prostate cancer or suffering cancer-related complications.

MATERIALS AND METHODS

Study Protocol

Patients were eligible to participate in the protocol at any of 15 medical centers if they had biopsy-proven untreated adenocarcinoma of the prostate with clinical stage T3N0M0 or T4N0M0 (bladder neck invasion alone) and were younger than 80-years-old. Clinical stage was confirmed according to UICC 1997 by digital rectal examination (DRE), transrectal ultrasonography (TRUS), chest X-ray, bone scan, abdominal-to-pelvic CT and pelvic MRI. Patients who were treated with antiandrogen or any adrenocortical steroid hormones, or had undergone subcapsular prostatectomy or transurethral resection of the prostate including laser ablation for benign prostatic hyperplasia, were

eliminated from this study. Pelvic MRI was conducted before or 3 months after prostate biopsy.

Patients were registered as primary candidates of the study and were treated with 2 weeks of steroidal antiandrogen (chlormadinone acetate; CMA), then with both luteinizing hormone-releasing hormone (LHRH) agonist (leuprorelin or goserelin) and another 2 weeks of antiandrogen, and thereafter with LHRH agonist alone. After 6 months of endocrine treatment with LHRH agonist, only patients with PSA levels lower than 10 ng/ml, with a PSA level lower than the pretreatment level and without clinically apparent metastatic disease were enrolled in the following protocol as final candidates (2nd-line registration). All Gleason scores were reviewed by one urologic pathologist (M.H.) before the 2nd-line registration. After the 2nd-line registration was done, the patients were randomly divided into two groups according to institutions, age (younger than 70, 70 years, or older), PSA levels after 6 months of endocrine treatment (4.0 ng/ml or lower, 4.1 ng/ml or greater), and Gleason score (7 or less, 8–10) as follows: (1) continuous androgen ablation group (arm 1), (2) intermittent androgen ablation group (hormonal therapy must be stopped 6 months after the day of final EBRT treatment) (arm 2) (Fig. 1). All of these patients were treated with EBRT immediately after completing 2nd-line registration.

Details on the procedures of radiation therapy were specified in the protocol as follows: (1) radiation field should be limited to the prostate in all cases, and the seminal vesicle should be included in radiation fields only in cases with seminal vesicle involvement being highly suspected by imaging. Elective pelvic lymph node irradiation is not performed. (2) Conformal radiation therapy, 4-field oblique or box technique, or pendulum methods are recommended in order to minimize adverse effects in the rectum and bladder. (3) A total dose of 72 Gy should be given in 36 fractions, 5 fractions per week. (4) Verification films should be taken at least two times during the radiation therapy. (5) The gross tumor volume (GTV) and clinical target volume (CTV) are the prostate gland in cases without seminal vesicle involvement. The planning target volume (PTV) margin is 10 mm from the CTV. In cases with seminal vesicle involvement, the GTV and CTV include the seminal vesicles in addition to the prostate gland. In multi-portal treatment, every portal should be irradiated in every treatment. (6) Only photon beam energy of 6 MV or more is accepted.

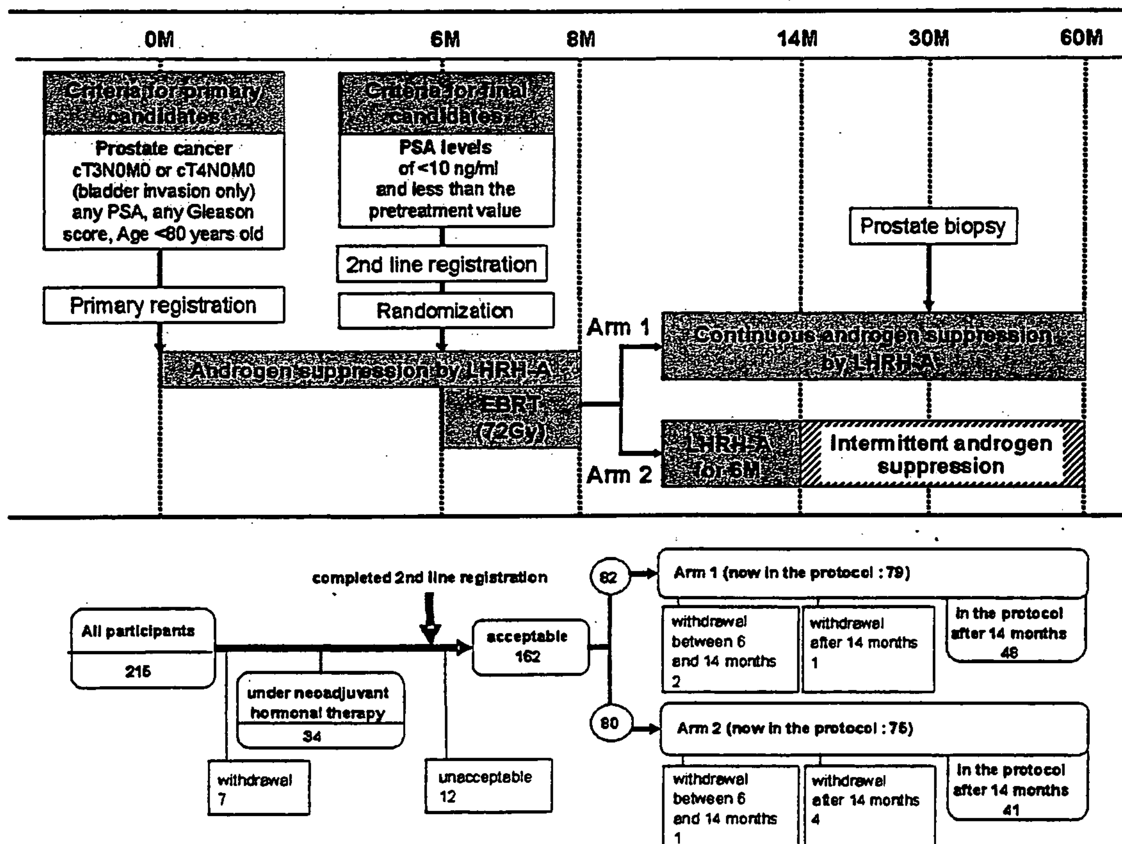

Fig. 1. Scheme of the study protocol, the number of patients registered and the present status of those patients in this study protocol. LHRH-A, LHRH agonist; EBRT, external beam radiation therapy.

Acute radiation morbidity should be evaluated by using common toxicity criteria of NCI within 90 days after radiation therapy, and late radiation morbidity should be evaluated by using the late radiation morbidity criteria of RTOG/EORTC.

Patients assigned to the intermittent androgen ablation group (arm 2) resumed hormonal therapy if they had PSA level of 10 ng/ml or greater or a clinical recurrence of disease. Resumed hormonal therapy would continue until the PSA levels decreased to below 1.0 ng/ml. If the PSA levels did not decrease to below 1.0 ng/ml, the possibility of biochemical recurrence of disease would be evaluated using the criteria in the study.

Biochemical failure was defined according to modified ASTRO criteria as follows: (1) three consecutive PSA increases in every 3-month interval and with a PSA velocity per 3 months of 0.5 ng/ml or greater, or (2) PSA levels increasing to 10 ng/ml or more. If three consecutive monthly-checked PSA levels increased rapidly at a PSA velocity per month of 0.17 ng/ml or greater, the researchers could designate that phenomenon a biochemical recurrence. The day of biochemical recurrence was defined between the day immediately before PSA increase and the day of initial PSA increase.

Clinical relapse was defined as progressive disease at a new site, an increase in the size of a nodule or cancer lesion on any images of the prostate, worse performance status, or body weight loss due to progression of prostate cancer.

Figure 2 shows the clinical assessment schedule of evaluation of treatment efficacy, QOL and adverse effects. PSA levels are measured monthly. Bone scan, abdominal-to-pelvic CT and chest X-ray must be conducted every 6 months for 1 year, and yearly thereafter. Pelvic MRI is conducted yearly. Prostate biopsy is recommended at around 2 years after the first date of EBRT. QOL can be assessed using FACT-P and part of the UCLA prostate cancer index before the initial endocrine therapy (0 months), immediately before EBRT (6 months), immediately after EBRT (8 months), 6 months after EBRT is completed (14 months), and 6 months after dividing the patients into two arms (20 months).

In the present study, treatment efficacy, adverse effects and QOL were compared between the two groups. The primary endpoint was biochemical (PSA) relapse-free survival. The secondary endpoints were overall survival, cause-specific survival, and longitudinal QOL assessment.

Cost effectiveness was also compared between men treated with continuous endocrine therapy and those with intermittent hormonal therapy.

The study protocol of this RCT and the documents of informed consent for the participants were approved by the IRB of all facilities, and a copy of the IRB approval document has been stored in the research bureau.

Statistical Consideration on Primary Endpoint of the Study

There has been no conclusive information on the optimal treatment strategy of adjuvant endocrine therapy after EBRT in patients with locally advanced prostate cancer. Therefore, the present study was conducted on the basis of the following two hypotheses. First, there was the non-recessive hypothesis, that the cumulative biochemical relapse-free survival rate in the intermittent endocrine therapy group (arm 2) would not be remarkably worse than that in the

Variables	Months after enrollment																				
	0	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54	57	60
PSA measurement	◎	← monthly check-up →																			◎
Digital rectal examination	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎
Transrectal ultrasonography	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎
Abdominal and pelvic CT	◎		◎		◎				◎				◎				◎				◎
Pelvic or endorectal MRI	◎				◎				◎				◎				◎				◎
Bone scintigraphy	◎		◎		◎				◎				◎				◎				◎
Chest X-P or Chest CT	◎		◎		◎				◎				◎				◎				◎
Prostate biopsy	◎										◎										◎
QOL assessment	◎		◎	◎(8M)		◎(14M)		◎(20M)													
Uroflowmetry	○				○				○				○				○				○
Residual urine	○				○				○				○				○				○
Blood test	◎	◎	◎	◎	◎		◎		◎		◎		◎		◎		◎		◎		◎
Performance status	◎				◎				◎				◎				◎				◎

◎ Essential assessment
○ Recommended assessment

Fig. 2. Assessment protocol for treatment effects, adverse effects and QOL in the study.

continuous endocrine therapy group (arm 1). If intermittent endocrine therapy after definitive EBRT is acceptable, the present study may be worthwhile from social, economic, and QOL points of view. The study would verify that the cumulative biochemical relapse-free survival rate in the continuous endocrine therapy group (arm 1) can be significantly better than that in the intermittent endocrine therapy group (arm 2). The second hypothesis was that continuous androgen suppression after EBRT may be worthwhile in terms of treatment efficacy, because of the specific characteristics of treatment for prostate cancer, which is famous for being hormone-naïve for a while in most cases. It would be possible to verify both of the above-mentioned hypotheses simultaneously by investigating the interval estimation of the hazard ratio, if the linearity can assume either hypothesis by carrying out the interval estimation of the hazard ratio, if the linearity can assume the recurrence hazard. Then, the 90% confidence interval for the hazard ratio (intermittent group/continuous group) can be calculated at both sides. If the upper limit is within the acceptable threshold, then the non-recessive hypothesis has been verified. On the other hand, the survival rate of the continuous group (arm 1) would be considered significantly excellent if the lower limit surpasses 1.

The main subjects for the analyses are qualified patients from whom the protocol treatments have been properly conducted. The analysis is limited to cases without remarkable contravention and deviation is carried out. The survival curve and recurrence-free survival will be estimated using Kaplan–Meier methods, and the confidence interval of the proportion at 3 and 5 years calculated by the formula of Greenwood. The hazard ratio is estimated by score statistic values from the log rank test results. Supplemental, by the hazard ratio is estimated by the Cox's proportional hazard model using the allocated factors at the 2nd registry, except for that of the facilities. The verification of the proportion hazard is done by double logarithm plotting, and the necessary analysis is carried out for the interpretation of results, such as the appliance of the Cox's proportional hazard model for time-dependent changes of the effects, when there is a remarkable dissociation from the proportion hazard. Prognostic factors which seem to be important are analyzed by means of each allocated factor at the 2nd registry except for that of the facilities, and the uniformity of differences between the two groups is examined. If necessary, the interaction between each facility and its remaining allocated factors at the 2nd registry will be analyzed, and also the differences between one facility and another.

The upper limits for the determination of non-recessiveness are 1.5 and 1.333. These upper limits may be acceptable if the hazard for combination treatment with EBRT and long-term endocrine therapy is outlining these thresholds compared with that for EBRT alone. These consequences have already been clarified by Bolla et al. [3], in which the confidence interval of hazard for disease-free survival was demonstrated between 1/0.15 and 1/0.32. According to the results of the Bolla study [3], an upper limit for the determination of non-recessiveness of 1.5 may be acceptable. On the other hand, the upper limit of 1.333 will also be used for an alternative analysis, because it may be a reference threshold for RCTs comparing treatment efficacy for other cancers.

Intermediate Assessment and the Possibility of Withdrawal of This Protocol

At the time when the number of enrolled cases reaches half of the expected adequate number of cases, an intermediate analysis will be performed to investigate whether the main purpose of the test has already been achieved, and another at the time when the expected adequate number of cases is fully registered. The intermediate analysis will be investigated blind by one statistician (Y.O.) at the registration center of the study in Tokyo University. If the disease-free survival in one group is significantly worse than that in the other group after careful consideration of the intermediate analysis, it will be decided whether the study protocol should continue or not.

Number of Cases Required for the Study, When to Close the Registration, and the Follow-Up Period

Considering that the cumulative PSA recurrence rate within 5 years in treatment with endocrine monotherapy for locally advanced prostate cancer in Japanese was demonstrated at about 40% [7], and that in combination therapy with EBRT and endocrine therapy was demonstrated between 15 and 64% [3,4], the cumulative PSA recurrence rate within 5 years in men treated with 3 years of adjuvant endocrine therapy and EBRT, in the present study, was assumed to be 30% [3]. For non-recessive verification using a hazard ratio of 1.5 as an upper limit, 75 events are necessary in each group in order to have 80% statistical power on the basis of the alternative hypothesis, in which there is no difference in the disease-free survival rate between both groups. Alternatively, on the basis of the alternative hypothesis which uses a hazard ratio of 2, the necessary event number for the dominance verification in both groups is 55, for 80% statistical power. There may be 90–100 events in 300 patients in the protocol during 5 years of observation. Therefore, if the cumulative disease-free survival rate in the continuous endocrine group is better with a hazard ratio of 2 or

more than that in the intermittent endocrine group, it may be possible to verify the dominance with high probability, which would be 93–95% if the number of the events is 90–100. Alternatively, if the cumulative rates for disease-free survival are similar between the two groups, pursuing non-recessive verification can not be avoided. In fact, the power decreases to 61–65% if there are 90–100 events.

It is worthwhile to consider that the significance of the study is the reevaluation by meta analysis with other clinical researchers around the world, who have almost the same hypothesis for verification, when non-recessiveness and dominance can not be verified. On the other hand, it is also possible to continue registration for another few years in some cooperative facilities, because randomization to one of two arms may be permitted even in the ethics target. Furthermore, it would also be possible to conduct a multi-factorial experiment, containing the LHRH administration period as a factor, and then performing a meta analysis.

The number of expected registered cases was set at 300 and the registration period 3 years in the protocol.

Patient Characteristics Registered

Between February 2001 and November 2003, 215 patients were registered in the protocol. Table I shows the clinicopathological features of patients registered in the present study. Age ranged from 54 to 79 years (70.6 ± 5.6, mean ± SD; 72.0, median). The median PSA level at entry was 25.3 ng/ml (45.1 ± 64.3; mean ± SD). The clinical stage was T3N0M0 in 202 (94.0%) and T4N0M0 in 13 (6.0%). The Gleason score diagnosed by the central urologic pathologist was 2–6 in 32 cases (16%), 7 in 94 cases (48%), and 8–10 in 68 cases (35%).

Details in the progression of this protocol in all participants are shown in Figure 1. On November 15, 2003, 188 patients (87.4%) were still in the protocol and 27 patients (12.6%) had withdrawn from the protocol. A total of 19, 3, and 5 cases were excluded from the protocol during 0–6 months, 6–14 months, and after 14 months of the protocol treatment, respectively. Of the 27 cases excluded from the protocol, 3 cases (11%) had adverse effects, 6 cases (22%) withdrew their agreement to this protocol, 1 case (4%) had other life-threatening cancer during the protocol treatment, 4 cases (15%) had recurrence of disease, 12 cases (44%) did not meet the criteria at the 2nd registration, and 1 case (4%) was excluded from the protocol by a contravention issue.

Of the 188 cases in the protocol, 34 patients (18%) received neoadjuvant hormonal therapy between 0 and 6 months of the protocol treatment, 64 patients (34%) were treated with EBRT and adjuvant endocrine therapy between 6 and 14 months, and 90 patients (48%) were treated with continuous or intermittent androgen ablation after 14 months of the protocol treatment.

Of the 95 cases who continued the protocol treatment after 14 months, 49 were treated with continuous endocrine treatment (arm 1) and 46 were treated with intermittent endocrine treatment (arm 2). The mean follow-up duration was 22.2 months (ranged from 14 to 30 months) in arm 1 and 23.0 months (ranged from 14 to 30 months) in arm 2. Of the 49 patients registered in arm 1, 1 case (2.0%) was excluded from the protocol because of recurrence of disease. Of the 46 cases registered in arm 2, 4 cases (8.7%) were excluded from the protocol treatment, because of recurrence of disease in 2 cases, contravention of the protocol in 1 case, and their own decision in 1 case.

TABLE I. Clinicopathological Features at Entry

Age	
Mean ± SD	70.6 ± 5.6
Median	72
Age distribution	
54–59	7 (3.3%)
60–64	28 (13.0%)
65–69	38 (17.7%)
70–74	80 (37.2%)
75–79	62 (28.8%)
PSA level (ng/ml)	
Mean ± SD	45.1 ± 64.3
Median	25.3
PSA distribution	
0.0–4.0	3 (1.4%)
4.1–10.0	38 (17.7%)
10.1–20.0	41 (19.1%)
20.1–50.0	79 (36.7%)
50.1–100.0	33 (15.3%)
100.1–∞	21 (9.8%)
Gleason score by (hospital pathologists)	
2–6	26 (12.1%)
7	106 (49.3%)
8–10	83 (38.6%)
Primary Gleason grade (hospital pathologists)	
–3	92 (42.8%)
4–5	123 (57.2%)
Clinical stage	
T3N0M0	202 (94.0%)
T4N0M0	13 (6.0%)
Gleason score by (central pathologist)	
2–6	32 (16.5%)
7	94 (48.5%)
8–10	68 (35.1%)
Primary Gleason grade (central pathologist)	
–3	99 (51.0%)
4–5	95 (49.0%)

RESULTS

Changes in the PSA levels within 1 month before prostate biopsy (pretreatment), after 6 months of endocrine treatment, 8 months of endocrine treatment (immediately after EBRT), and 14 months of endocrine treatment (6 months after EBRT) are shown in Table II. The PSA levels showed a remarkable decrease to median (mean ± SD) levels of 1.1 ng/ml (2.7 ± 5.0), 0.2 ng/ml (0.6 ± 1.0) and 0.1 ng/ml (0.3 ± 0.5) after 6, 8, and 14 months of the protocol treatment, respectively. The proportion of patients with PSA levels of 1.0 ng/ml or lower was 49% (85/173), 81% (118/145), and 91% (86/95) at 6, 8, and 14 months of the protocol treatment.

Of the 157 cases treated with EBRT, excluding eliminated cases without recurrence of disease, 153 cases (97.5%) had no biochemical failure in the mean follow-up of 17.3 months (range from 6.7 to 34.3 months).

A total of 44 cases were treated by intermittent hormonal therapy. Of the 44 cases, 41 cases have had no endocrine treatment according to the criteria after 14 months of the protocol treatment. Of the 401 months of the post-intermittent phase (i.e., after 14 months in the protocol treatment), in all 44 cases, 394 months (98.3%) were without treatment with endocrine therapy according to the criteria (off-treatment).

Of the 44 cases within the intermittent treatment protocol, 3 cases (6.8%) resumed endocrine therapy, because of clinical progression in 1 case and PSA levels increasing to greater than 10 ng/ml in 2 cases.

DISCUSSION

Although the treatment efficacy of intermittent endocrine therapy has not been clarified, it would be expected to have significance in the QOL, cost and prevention of decreasing bone mineral density. Several investigators have demonstrated the possibility of the clinical utility of intermittent endocrine therapy. The proportion of off-treatment periods were 38–50% during 24–30 months of follow-up periods in men with prostate cancer treated with endocrine monotherapy [8–10]. Most of the non-randomized trials have reported a response to the reintroduction of hormonal therapy in 90% of patients, with an on-treatment/off-treatment ratio of about 40–60% [8,11–17]. However, there had been no RCT to investigate the possibility of intermittent endocrine therapy in combination with EBRT in men with locally advanced prostate cancer. The biochemical recurrence rate may be higher in men treated with intermittent endocrine therapy than in those with continuous endocrine therapy. However, additional EBRT may improve disease-free survival for men with locally advanced prostate cancer. The present study revealed that the on-treatment/off-treatment ratio was extremely low at 1.8%. Therefore, the present RCT can solve uncertainties of treatment efficacy and QOL for intermittent endocrine therapy in combination with EBRT for men with locally advanced prostate cancer.

In the present study, disease-free survival was defined as a primary endpoint, because a previous study demonstrated a high 5-year overall survival rate of 92% and a relatively low 5-year biochemical disease-free survival rate of 61% in patients with locally advanced prostate cancer treated with LHRH agonist alone [7]. To set biochemical disease-free survival as the primary endpoint, it may be possible to have enough statistical power during a 5-year follow-up. The validity of this setting may be acceptable, because there is a limitation to the treatment after developing hormone-insensitive prostate cancer. Furthermore, any endocrine treatments will not be effective after recurrence of disease and the life span may be limited.

TABLE II. Changes in the PSA Levels After 6, 8, and 14 Months of the Protocol Treatment

	0 month	6 months	8 months	14 months
n	215	173	145	95
PSA level (ng/ml)				
Mean ± SD	45.1 ± 64.3	2.7 ± 5.0	0.6 ± 1.0	0.3 ± 0.5
Median	25.3	1.1	0.2	0.1
PSA distribution				
0.0–1.0	0 (0.0%)	85 (49.1%)	118 (81.4%)	86 (90.5%)
1.1–2.0	0 (0.0%)	29 (16.8%)	14 (9.7%)	9 (9.5%)
2.1–4.0	3 (1.4%)	33 (19.1%)	11 (7.6%)	0 (0.0%)
4.1–10.0	38 (17.7%)	15 (8.7%)	2 (1.4%)	0 (0.0%)
10.1–20.0	41 (19.1%)	6 (3.5%)	0 (0.0%)	0 (0.0%)
20.1–50.0	79 (36.7%)	5 (2.9%)	0 (0.0%)	0 (0.0%)
50.1–100.0	33 (15.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
100.1–∞	21 (9.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

The rates of biochemical no evidence of disease (bNED) control for patients with stage T3/T4 disease treated with a conventional dose of radiation therapy alone are poor, between 25 and 32% at 5 years [18,19] and 37% at 6 years [20]. The 5-year bNED in patients treated with EBRT alone for stage T1 to T4 disease decreased as pretreatment PSA levels increased, that is a bNED of 82–100%, 44–66%, 27–72%, and 11–14% for patients with pretreatment PSA levels of 4 ng/ml or less, 4–10 ng/ml, 10–20 ng/ml, and greater than 20 ng/ml, respectively [18,20–22]. The bNED control rate is higher in men treated with 3DCRT than in those treated with conventional EBRT even for cases with high levels of PSA. However, the bNED at 5 years is still low at 75 and 32% in patients treated with a high radiation dose of 76 Gy, in the PSA range of 10–20 ng/ml and greater than 20 ng/ml, respectively [23]. These treatment failures might result from the limitation of EBRT for large volume cancer on one side and the existence of clinically undetectable metastasis on the other side.

These poor outcomes of EBRT for locally advanced prostate cancer led to several randomized controlled trials on the effectiveness of neoadjuvant or adjuvant hormonal therapy in comparison with EBRT alone by the Radiation Therapy Oncology Group (RTOG) and The European Organization for Research and Treatment of Cancer (EORTC).

The RTOG 86-10 was conducted to investigate the usefulness of androgen ablation 2 months before and during EBRT compared with EBRT alone for locally advanced prostate cancer [5]. The biochemical disease-free survival and cause-specific mortality were significantly better in men undergoing androgen ablation before and during EBRT than in those treated with EBRT alone, especially in patients with Gleason 2–6 tumors.

Bolla et al. [3] conducted an RCT comparing overall survival and the disease-free interval between men treated with EBRT alone and with EBRT in combination with 3 years of adjuvant endocrine therapy starting from the initial date of EBRT (EORTC 22863) [3]. They demonstrated that the 5-year overall survival rate was significantly higher at 79% in patients treated with combination therapy than that in those treated with EBRT alone, which was 62%. The 5-year disease-free survival rate was also significantly higher at 81% in patients treated with combination therapy than that in those treated with EBRT alone.

The effectiveness of adjuvant endocrine therapy in combination with EBRT for patients with locally advanced prostate cancer can be clarified. Although cancer volume may be a very important factor in the treatment of EBRT, clinical data addressing the potential value of hormonal cytoreduction before radiotherapy have been quite limited. Therefore, it can also be valuable to investigate whether neoadjuvant endocrine therapy before EBRT is useful for locally advanced prostate cancer. In the present study protocol, all patients were initially treated with endocrine therapy for 6 months, and only patients with PSA levels after 6 months of endocrine therapy of 10 ng/ml or lower and also lower than the pretreatment levels were enrolled as final candidates in this study. The eliminated cases without sufficient effects after 6 months of endocrine treatment should be treated with other treatment protocols like chemoendocrine treatment. Therefore, our study protocol, which selects only patients with sufficient effects by neoadjuvant endocrine treatment, may be acceptable by means of ethical issues and also scientific validity.

At present, EBRT in combination with adjuvant endocrine therapy for locally advanced prostate cancer can be recommended in terms of survival benefit. However, it has not been clarified when and how long additional endocrine therapy should be conducted with respect to not only survival but also QOL. The compliance of this RCT may be high, so it is expected that long-term follow-up of the participants in the present study will reveal the possibilities of intermittent endocrine therapy after EBRT in patients with locally advanced prostate cancer.

Members of the National Research Project on Endocrine-Radiation Combination Therapy for Locally Advanced Prostate Cancer

Hidetoshi Yamanaka, Hiroyuki Fujimoto, Naoki Matsuoka, Taiji Tsukamoto, Iwao Fukui, Seiji Naito, Michiyuki Usami, Kazuhiro Suzuki, Norio Mitsuhashi, Tetsuo Akimoto, Minako Sumi, Masato Hareyama, Takashi Yamashita, Katsumasa Nakamura, Koichi Tokuuye, Kinji Nishiyama, Yasuo Ohashi, Masaoki Harada, Jun Aoki, Mitsuru Shinohara, Katsuyuki Karasawa, Satoshi Kitahara, Miwako Nozaki, Shin Egawa, Iku Nishiguchi, Masashi Kitano, Takanori Suzuki, Nobuaki Shimizu, Yoshio Tamaki, Mikio Kobayashi, Iku Takahashi, Mikinobu Ohtani, Akio Iwasaki, Tatsuo Tochigi, Shiro Saito, Katsuyoshi Hashine, Tadao Kakizoe, Toshihiko Kotake.

Associate Researchers in the Project

Kazuto Ito, Naoya Masumori, Norio Meguro, Hirofumi Koga, Sadafumi Kawamura, Kotaro Gomi, Yutaka Takezawa, Atsushi Yamauchi, Takumi Yamamoto, Akira Irie, Kazumi Shiojima.

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Original

Lack of Chemoprevention or Promotion Effects of Docosahexaenoic Acid on Small Intestine, Colon, Liver, Lung, Thyroid, Esophagus, Kidney, and Forestomach Carcinogenesis in a Rat Medium-Term Multi-Organ Carcinogenesis Model

Toshio Ichihara[1,2], Seiko Tamano[1,2], Hiroko Yoshino[1,2], Katsumi Imaida[3], Hideki Ishikawa[4], Tadao Kakizoe[5], and Tomoyuki Shirai[1]

[1]Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467–0001, Japan
[2]DIMS Institute of Medical Science, 64 Goura, Nishiazai, Azai-cho, Ichinomiya 491–0113, Japan
[3]Onco-Pathology, Faculty of Medicine, Department of Pathology and Host-Defense, Kagawa University, 1750–1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761–0793, Japan
[4]Laboratory of Hereditary Tumor, Institute for Advanced Medical Sciences, Hyogo College of Medicine, 2–3–1–2F Kyomachibori, Nishi-ku, Osaka, 550–0003, Japan
[5]National Cancer Center Hospital, 5–1–1 Tsukiji, Chuo-ku, Tokyo 104–0045, Japan

Abstract: Modifying effects of docosahexaenoic acid (DHA) were examined using a medium-term multi-organ carcinogenesis model (DMBDD model). Groups of twenty F344 male rats were treated sequentially with *N*-diethylnitrosamine (DEN, i.p.), *N*-methyl-*N*-nitrosourea (MNU, i.p.), 1,2-dimethylhydrazine (DMH, s.c.), *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN, in drinking water) and dihydroxy-di-*N*-propylnitrosamine (DHPN, in drinking water) during the first 4 weeks (DMBDD treatment), and then DHA-ethyl ester (DHA-E), DHA-triglyceride (DHA-TG) and/or tocopherol were administered intragastrically 3 times a week for 31 weeks. Significant inhibition of the development of glutathione *S*-transferase placental form (GST-P) positive foci was observed in DMBDD treated 30% DHA-TG 404 mg and 128 mg + tocopherols groups and with tocopherol alone; however, this appeared to be due to the tocopherol. DHA treatment did not influence the development of aberrant crypt foci in the large intestine. Histopathologically, the incidences of preneoplastic and neoplastic lesions in other organs were also not increased or decreased by DHA treatment. Thus, the results indicate a lack of chemopreventive and tumor promotion effects of any type of DHA in male rats under the present experimental conditions. (J Toxicol Pathol 2005; 18: 53–59)

Key words: docosahexaenoic acid, medium-term multi-organ carcinogenesis model, F344 rat, promotion

Introduction

The n-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA) is a major component of fish oil, which has been frequently reported to have chemopreventive potential for colon, mammary gland and pancreas carcinogenesis in rats[1–6]. For example, DHA was found to suppress aberrant crypt foci (ACF) in the colon induced by azoxymethane (AOM) or 1,2-dimethylhydrazine (DMH)[1,3]. Furthermore, induction of ACF by the heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[*4,5-b*]pyridine (PhIP), was also inhibited by DHA treatment[4]. Furthermore colon cancer multiplicity was significantly decreased in another study[2,3]. In the mammary gland, development of tumors was also reduced by a low dose of DHA or eicosapentaenoic acid (EPA) treatment after carcinogen (DMBA) injection[6]; however, in a clinical trial with familial adenomatous polyposis (FAP) patients a high risk group for colorectal cancer, it was without major influence[7]. The three FAP patients were administered concentrated DHA in fish oil capsules (2.2 g of DHA-TG and 0.6 g eicosapentaenoic acid (EPA) per day) for one or two years. The patients with FAP developed endometrial cancer after 12 months, colon cancer after 24 months and lung cancer after 12 months, respectively[7].

It is well established that a chemical may act as a tumor inhibitor in one organ and as a promoter in others[8–10]. It is

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Mailing address: Toshio Ichihara, DIMS Institute of Medical Science, 64 Goura, Nishiazai, Azai-cho, Ichinomiya 491–0113, Japan
TEL: 81-586-51-1201 FAX: 81-586-51-5634
E-mail: ichi@dims.co.jp

Table 1. Fatty Acid Contents for a Rat in Dosing Solvent (mg)

Groups		16:0 Palmitic acid	18:0 Stearic acid	18:1 Oleic acid	18:2 Linoleic acid	20:1 Gadoleic acid	20:4(n-6) AA	20:5 EPA	22:5 DPA	22:6 DHA	Other FA	tocopherol	Total
1,6	128 mg 97% purify DHA-E	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	120.0	4.0	4.0	128.0
2,7	404 mg 30% purify DHA-TG	56.8	13.6	74.0	5.2	10.0	8.0	32.8	12.0	113.2	74.4	4.0	404.0
3	128 mg 30% purify DHA-TG	17.6	4.2	22.9	1.6	3.1	2.5	10.2	3.7	35.1	23.1	4.0	128.0
4	4 mg tocopherol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.0	4.0

AA: arachidonic acid. EPA: eicosapentaenoic acid. DPA: docosapentaenoic acid. FA: Fatty acid.

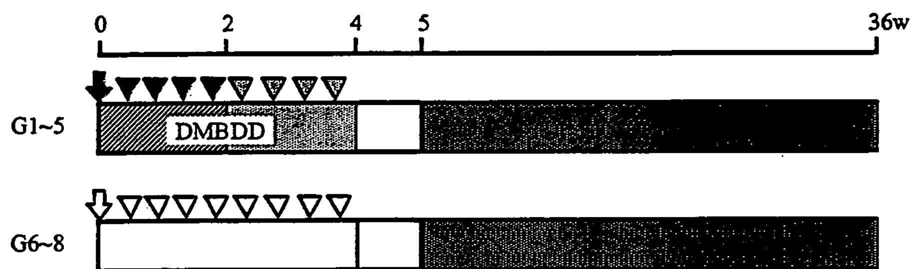

Fig. 1. Experimental protocol for the medium-term multi-organ carcinogenesis model. Animals: male F344/DuCrj rats, 6 weeks old; ↓, DEN, 100 mg/kg body wt. i.p.; ▼, MNU, 20 mg/kg body wt. i.p.; ▽, DMH, 40 mg/kg body wt. s.c.; ▨, BBN, 0.05% in drinking water; ▒, DHPN, 0.1% in drinking water; ⇩▽, saline injection; ■, G1 and 6, 128 mg 97% purity DHA-E, G2 and 7, 404 mg 30% purity DHA-TG, G3, 128 mg 30% purity DHA-TG, G4, 4 mg tocopherol, G5 and 8, no treatment.

therefore important to examine modification potential not in a single organ, but rather in the whole body. This requires *in vivo* experimental models which can detect effects in a wide spectrum of organs, and for this purpose several multi-organ wide-spectrum initiation models have been established[11–14]. The medium-term approach has clear benefits for the examination of modifying effects of chemicals in multiple organs in a single experiment within a relatively short experimental period[15–17] and is based on the proven good agreement between the multi-organ carcinogenesis model and long-term experimental results[18].

The ethyl ester formed by DHA (DHA-E) has been used in many chemoprevention studies[1–6], and DHA-TG has been used in a clinical trial study[7]. Therefore, we thought it important to investigate the difference in the modifying effects on carcinogenesis of DHA-E and DHA-TG. In the present study, we investigated the post-initiation-phase modifying activity of DHA-E and DHA-TG at the whole organ level using a rat medium-term multi-organ carcinogenesis model developed in our laboratory[8,15,19,20]. Furthermore, a tocopherol group was included as a comparative control.

Materials and Methods

Animals

Male F344 rats, aged 5 weeks, were obtained from Charles River Japan Inc. (Kanagawa Japan), and housed five to a plastic cage with wood chips for bedding in an air-conditioned room at 22 ± 2°C with a 12-h light: 12-h dark cycle. They were maintained on Oriental MF diet (Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*. The study was started after 1 week of acclimatization.

Chemicals

N-Diethylnitrosamine (DEN), *N*-methyl-*N*-nitrosourea (MNU), 1,2-dimethylhydrazine (DMH) and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and dihydroxy-di-*N*-propylnitrosamine (DHPN) was obtained from Nacalai Tesque Co. (Osaka, Japan). The DHA dosing solution was supplied by Nippon Suisan Kaisha, Ltd. (Tokyo, Japan). DHA naturally exists in fish oil as a triglyceride (DHA-TG). DHA-E was chemically synthesized from DHA-TG by removing other fatty acids such as oleic acid and EPA. The contents in the dosing solution used in the present study are shown in Table 1. They were stored in sealed ampules under anaerobic conditions at –20°C in the dark.

Experimental methods

Medium-term multi-organ carcinogenesis study

The experimental protocol is shown in Fig. 1. The animals were randomly allocated to 8 groups of 10 – 20 animals. Those in groups 1 to 5 received the combined carcinogen treatments, consisting of a single *i.p.* injection of 100 mg/kg body wt. of DEN, four *i.p.* injections of 20 mg/kg body wt. of MNU, four *s.c.* injections of 40 mg/kg body wt.

Table 2. Final Body and Organ Weights Data

Groups	DMBDD	Treatment	Effective no. of rats	Body wt.[a)] (g)	Liver wt.[a)] (g)	Liver wt.[a)] (%, b.w.)	Kidneys wt.[a)] (g)	Kidneys wt.[a)] (%, b.w.)
1	+	128 mg 97% DHA-E	19	312.4 ± 14.8[b)]	6.60 ± 0.45	2.11 ± 0.08	2.00 ± 0.44	0.64 ± 0.15
2	+	404 mg 30% DHA-TG	17	318.6 ± 15.5	7.01 ± 0.51	2.23 ± 0.11[c)d)]	3.12 ± 3.15	1.00 ± 1.00
3	+	128 mg 30% DHA-TG	19	306.8 ± 20.5[c)]	6.53 ± 0.59	2.13 ± 0.09	2.03 ± 0.65	0.67 ± 0.25
4	+	4 mg Tocopherol	18	314.8 ± 24.7	6.74 ± 0.54	2.14 ± 0.10	1.97 ± 0.22	0.62 ± 0.06
5	+	no treatment	19	324.6 ± 18.2	6.83 ± 0.47	2.10 ± 0.08	2.03 ± 0.36	0.63 ± 0.11
6	–	128 mg 97% DHA-E	10	356.6 ± 18.2	7.56 ± 0.71	2.13 ± 0.24	1.93 ± 0.16	0.54 ± 0.02
7	–	404 mg 30% DHA-TG	10	373.0 ± 11.7	7.80 ± 0.24	2.09 ± 0.06	2.05 ± 0.11	0.55 ± 0.03
8	–	no treatment	10	369.5 ± 14.9	7.61 ± 0.36	2.06 ± 0.05	2.00 ± 0.11	0.54 ± 0.03

a) Mean ± SD.
b), c) Significantly different from group 5 at $P<0.05$ and 0.01, respectively.
d) Significantly different from group 4 at $P<0.05$.

of DMH, together with 0.05% BBN for 2 weeks and then 0.1% DHPN for 2 weeks (both given in the drinking water), during the initial 4 week period for multiple initiation (DMBDD treatment) as described previously[21-23]. Animals in groups 1 to 5 were then given intragastric injections, 1 ml of 128 mg/ml of 97% purity DHA-E, 404 mg/ml of 30% purity DHA-TG, 128 mg/ml of 30% purity DHA-TG, each with 4 mg/ml of tocopherol, or tocopherol alone or distilled water, 3 times a week from 1 week after completion of the DMBDD treatment to the end of the experiment. Animals in groups 6 to 8 were given 128 mg/ml 97% purity DHA-E, 404 mg/ml 30% purity DHA-TG and distilled water as a solvent control without DMBDD treatment from week 5. The treatment times per week and concentration of DHA dosing solution were decided according to a trial study[7]. Animals were weighed once a week in the initial 14 weeks, then once every 2 weeks until the end of the study period, at week 36, when all surviving animals were sacrificed by exsanguination under ether anesthesia and subjected to complete necropsy.

All experimental procedures were performed in accordance with the in-house guideline for the Care and Use of Laboratory Animals at DIMS Institute of Medical Science.

Aberrant crypt foci assay

Nine or 10 rats for each treatment with DMBDD initiation and 5 rats each without DMBDD were analyzed for colon ACF. The colon was removed, slit open from the anus to the cecum along the longitudinal axis, flattened between sheets of filter paper, and fixed in buffered 10% formalin. Then it was stained with 0.2% methylene blue solution by the procedure of Bird[24] to observed aberrant crypts. The number of aberrant crypt foci per colon, the number of aberrant crypts in each focus, and the location of each focus was determined by microscopy.

Histopathological examination

At necropsy, the brain, liver, kidneys, spleen, heart, lungs, thymus, testes and adrenals were excised and weighed, and the relative percentage organ weights were calculated on the basis of final body weights. These and the other major organs including small and large intestines were fixed in 10% buffered formalin, and routinely processed. Paraffin-embedded sections were stained with hematoxylin and eosin for histopathological examination. Liver slices fixed in 10% buffered formalin were also prepared for quantitative assessment of immunohistochemically demonstrated glutathione *S*-transferase placental form (GST-P) positive foci, as previously described[25]. GST-P positive foci larger than 0.2 mm in diameter and the total areas of the liver sections examined were quantitated using a video image processor (SPICCA-II, Nippon Avionics, Tokyo, Japan) and the data expressed as numbers and areas (mm^2) per unit area of the liver section (cm^2).

Statistical analysis

The significance of intergroup differences in numerical data obtained for body and organ weights was assessed using the two-tailed Student's t-test. Insufficient homogeneity of variance was corrected with respect to the degrees of freedom according to the method of Welch. The significance of differences in the incidences of histopathological findings between treated and control groups was evaluated using Fisher's exact probability test.

Results

No post-initiation treatment-related clinical signs or mortalities were noted in any of the groups in the current experiment. Eight rats were found dead in the course of study, one in group 1, three in group 2, one in group 3, two in group 4 and one in group 5, and the deaths were all considered to have been caused by the DMBDD treatment.

The average body weights of rats in the DMBDD treated groups were significantly less than in the non-DMBDD initiated groups, throughout the study period. After DMBDD initiation, 30% DHA-TG was associated with retardation of body weight increase from week 7. The body weights in the other DMBDD treated groups were not