discrepancy and are thus preparing a rat study to examine the effect of ERβ on VSMC proliferation in vivo.

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Results of a phase I clinical study using autologous tumour lysate-pulsed monocyte-derived mature dendritic cell vaccinations for stage IV malignant melanoma patients combined with low dose interleukin-2

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We conducted a pilot study to assess the feasibility and efficacy of immunotherapy for stage IV malignant melanoma patients resistant to conventional therapies involving vaccination with mature dendritic cells (mDCs) combined with administration of low dose interleukin-2. Autologous monocytes were harvested from a single apheresis and cultured for 7 days with granulocyte–macrophage colony-stimulating factor and interleukin-4, yielding immature dendritic cells (iDCs), which were then cryopreserved until use. For 4 days prior to vaccination, iDCs were exposed to autologous tumour lysate combined with tumour necrosis factor-α to induce terminal differentiation into mDCs. Patients were then vaccinated weekly with 10^7 mDCs for 10 weeks and given 350–700 kIU of interleukin-2 three times per week. Of the 10 patients in the study, one showed stable disease, seven showed progressive disease, and two showed mixed responses, including partial tumour regression, and were therefore given 20 additional injections. Only minimal adverse events were noted, including localized skin reactions and mild fever (NIH-CTC grade 0–1). Median survival from the first vaccination was 240 days (range 31–735 days). *In vitro*, melanoma patient-derived dendritic cells (DCs) showed reduced cell surface expression of CD1a antigen on iDCs and reduced CD86 and HLA-DR expression on mDCs. In addition, antigen uptake, chemotaxis and antigen presentation were all attenuated in DCs from the patients. In summary, although improvement of clinical efficacy will require further research, autologous tumour lysate-pulsed monocyte-derived mDCs could be safely harvested, cryopreserved and administrated to patients without obvious complications. *Melanoma Res* 13:521–530 © 2003 Lippincott Williams & Wilkins.

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Introduction

Dendritic cells (DCs) are unique major specialist antigen-presenting cells (APCs) capable of stimulating naive T-cells during primary immune responses more potently than either peripheral blood monocytes/macrophages or B-cells [1]. Previous studies have shown that immature DCs (iDCs) exhibit several characteristic features, including (i) vigorous endocytotic ability; (ii) a high capacity to produce pro-inflammatory cytokines; and (iii) potent chemotactic responses to inflammatory chemokines such as regulated on activation normal T-cell expressed and secreted (RANTES) and macrophage inflammatory protein (MIP)-1α via chemokine receptors CCR-1 and CCR-5, respectively [1–3]. The development of iDCs into mature DCs (mDCs) can be induced by a variety of stimuli, including bacterial components such as lipopolysaccharide, pro-inflammatory cytokines such as tumour necrosis factor-α (TNFα) and cognate CD4+ T-cell help via interaction with CD40/CD40 ligand. These events are accompanied by proteolytic cleavage within phagolysosomes, presentation of antigens at the cell surface by major histocom-

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patibility complex (MHC) proteins, upregulation of co-stimulatory molecules, the ability to stimulate T-cells, downregulation of the internalization of exogenous soluble antigen, and production of pro-inflammatory cytokines [1–3]. This maturation process also involves the downregulation of the cell surface expression of CCR-1 and CCR-5, resulting in diminished chemotactic responses to inflammatory chemokines, and enhanced expression of CCR-7, resulting in mDCs homing into secondary lymphoid tissues via the interaction of CCR-7 with MIP-3β, where they prime naive T-cells and initiate primary immune responses [1–3].

Evidence from both humans and animal models suggests that by enhancing tumour-specific T-cell responses, DCs actively contribute to a protective immunity against cancer [1]. Unfortunately, the use of DC-based tumour vaccines in clinical applications has been limited by the fact that there are relatively few DCs present in peripheral blood and other tissues, making isolation of sufficient cells for vaccination difficult [1–4]. Recently, however, a method was developed to generate DCs from peripheral blood, *in vitro*, by culturing their progenitor cells in cytokine-driven systems, a procedure that has profoundly changed preclinical research as well as the clinical evaluation of these cells [5,6]. Indeed, several groups have reported that vaccination with peripheral blood monocyte-derived iDCs or mDCs pulsed with tumour lysate or a cocktail of peptides derived from tumour-associated antigens (TAAs) appears to be potentially useful as an antitumour immunotherapy, although the clinical effectiveness and the impact on the survival of tumour patients remains unclear [7–9].

In this report, we describe a clinical pilot study in which vaccination with autologous, tumour lysate-pulsed, monocyte-derived mDCs combined with administration of recombinant human (rh) interleukin-2 (IL2) was used as immunotherapy to treat stage IV malignant melanoma patients resistant to conventional therapy. In addition, we also examined the immunological features of these DCs derived from peripheral blood monocytes from cancer patients to assess their suitability for the preparation of a DC-based tumour vaccine.

Materials and methods

Patient selection

This study protocol was approved by the Institutional Review Board of the Institute of Medical Science, University of Tokyo, Japan. All eligible patients were histologically proven to have melanoma with distant metastases. Ten patients (five males, five females; aged 24–75 years, mean 47 years) who had previously tried various other therapies entered the study (Table 1). No alternative therapy was given. Inclusion criteria were an

Table 1 Patient characteristics, status before DC vaccination and response to DC vaccination

Patient no.	Age (years)	Sex	Status before vaccination					Response to DTH skin test			Survival (days from first vaccination)
			Site of primary tumour	Previous therapy	Sites of metastases	No. of DC vaccinations	Clinical response	Prevaccination	5 weeks	10 weeks	
1	24	Male	Skin	Surgery, chemotherapy	Liver, lymph node	6	PD	ND	ND	ND	59
2	27	Female	Skin	Surgery, chemotherapy	Skin, lymph node, lung	7	PD	±	+	ND	50
3	48	Female	Skin	Surgery, chemotherapy	Brain, liver, stomach, lymph node, lung	10	PD	±	+	+	328
4	24	Female	Unknown	None	Skin, lymph node, multiple organs (brain, lung, liver, ovary, etc.)	10	PD	±	ND	+	152
5	75	Male	Skin	Surgery, chemotherapy	Skin, lymph node	10	PD	ND	+	+	629
6	74	Male	Skin	Surgery, chemotherapy	Skin, adrenal, brain, orbit	10	SD	ND	+	+	342
7	35	Male	Skin	Surgery, chemotherapy	Skin, muscle, lung, pancreas, pelvis	8	PD	+	–	ND	52
8	58	Female	Unknown	Surgery, chemotherapy	Lymph node, skin, pericardium, retroperitoneum	30	MR	–	–	–	561
9	57	Male	Skin	Surgery, chemotherapy	Lung, muscle, adrenal	30	MR	–	±	ND	735
10	43	Female	Skin	Surgery, chemotherapy	Skin, bone, dura mater	5	PD	–	±	–	31

PD, progressive disease; SD, stable disease; MR, mixed response; ND, not done.

European Cooperative Oncology Group (ECOG) score of 0–2, adequate hepatic and renal function (total bilirubin < 2 mg/dl, serum creatinine < 2 mg/dl) and a life expectancy of more than 12 weeks. Patients with severe cardiac, pulmonary or psychiatric disease, or with acute uncontrollable infection, were excluded. As a control, peripheral blood samples from 12 normal healthy volunteers (five males, seven females; aged 23–79 years, mean 43 years) were collected and analysed. All of the study participants gave signed informed consent according to the Declaration of Helsinki before enrolling in the study.

Media and reagents

The medium used throughout was RPMI 1640 (Sigma, St Louis, Missouri, USA) supplemented with antibiotic-antimycotic (Gibco BRL, Gaithersburg, Maryland, USA) and 5% heat-inactivated human serum type AB (BioWhittaker, Walkersville, Maryland, USA). Recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF) was kindly provided by Kirin Brewery (Tokyo, Japan); rhIL2, rhIL4, rhTNFα, rhRANTES and rhMIP-3β were purchased from PeproTech (London, UK).

In vitro generation and culture of iDCs from stage IV malignant melanoma patients and normal healthy volunteers

iDCs were generated from leukapheresis products as described elsewhere [10–12]. Briefly, peripheral blood mononuclear cells (PBMCs) were collected from single 15 l leukaphereses from the patients and healthy volunteers using a COBE SPECTRA (Cobe Laboratories, Lakewood, Colorado, USA). The collected cells were then separated by density gradation using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden), and the light density fraction from the 42.5–50% interface was recovered. The cells were then resuspended in cold phosphate buffered saline (PBS) and allowed to adhere to 10 cm plastic dishes (Primaria, Becton Dickinson, Mountain View, California, USA) for 30 min at 37°C, after which non-adherent cells were removed and the remaining adherent cells were collected for DC preparation. The collected cells ($> 10^8$ cells/apheresis) were > 95% pure, as indicated by anti-CD14 monoclonal antibody (MAb) staining (BD Pharmingen, San Diego, California, USA).

To prepare the iDCs, the cells were cultured in 10 cm plastic dishes ($> 10^7$ cells/dish) with 6 ml of medium containing rhGM-CSF (final concentration 50 ng/ml) and rhIL4 (250 ng/ml) for 7 days at 37°C under a humidified atmosphere of 5% CO_2 in air, after which the resultant iDCs were harvested. Phenotypical analysis of the iDCs was carried out using a FACS Calibur flow cytometer (Becton Dickinson), CELL Quest Software (Becton Dickinson) and fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated isotype-matched control MAbs (BD Pharmingen). DC family markers (CD1a and CD11c), co-stimulatory molecules (CD40, CD80 and CD86) and HLA-DR were detected in iDCs from both melanoma patients and healthy donors. To assess the endocytotic activity of the iDCs, mannose receptor-mediated endocytosis of FITC-conjugated dextran (FITC-DX) (Molecular Probes, Eugene, Oregon, USA) and macropinocytosis of lucifer yellow (LY) (Molecular Probes) via cytoskeleton-dependent fluid-phase endocytosis were evaluated [10,11]. Chemotaxis of DCs to RANTES or MIP-3β (100 ng/ml) was assessed as described previously [10,11]. Data were expressed as the number of migrated cells/high power field (HPF).

Freezing and thawing procedures

The washed and counted iDCs were diluted in ice-cold medium consisting of 50% human type AB serum, 10% dimethyl sulphoxide and 40% RPMI 1640. The cells were distributed in 1 ml aliquots ($> 10^7$ cells) into pre-cooled plastic vials, after which the vials were placed in an isopropanol-containing biofreezing vessel (BICELL, Nihon Freezer, Japan) and the temperature was reduced to −135°C at a rate of 1°C/min. The cryopreserved samples were then stored until used.

For experimentation, the frozen vials were quickly thawed in a 37°C water bath, after which the cells were washed twice in washing medium consisting of 10% human type AB serum, 5% dextran and 85% RPMI 1640, plated in 10 cm plastic dishes, and cultured in the absence of cytokine for 24 h at 37°C. Preliminary studies showed that frozen and thawed samples contained greater than 95% viable cells as determined by trypan blue exclusion (data not shown).

Tumour lysate extraction

Tumour lysates were used as a tumour antigen since TAA-derived antigenic peptides suitable for our melanoma patients were not available. To prepare the lysates, tumour mass was obtained by exclusion of non-malignant tissues from tumour biopsies with a scalpel. The isolated mass was then homogenized, and tumour cells were obtained by depleting lymphocytes from the total cell suspension using immunomagnetic bead-conjugated MAbs against CD2, CD14 and CD19 (Dynal, Oslo, Norway) according to the manufacturer's instructions. Aliquots of the isolated tumour cells ($> 10^7$ = melanoma cells/tube) were then lysed by putting them through three freeze (in liquid nitrogen) and thaw (in a 37°C water bath) cycles. The lysed cells were centrifuged at 800 *g* for 5 min, and the supernatants were passed through a 0.22 μm filter (Millipore Corporation, Bedford, Massachusetts, USA). The protein contents of the resultant cell-free lysates were determined using DC protein assay kits (Bio-Rad Laboratories, Hercules,

California, USA). Aliquots (500 μg/tube) were then stored at −135°C until use.

Preparation of tumour lysate-pulsed mDCs for vaccination

iDCs (> 10^7 cells/dish) in 10 cm plastic dishes were cultured for 24 h in 3 ml of medium containing 100 μg/ml of autologous tumour lysates, and then in 6 ml of medium containing 50 ng/ml rhTNFα for an additional 4 days to induce terminal differentiation into mDCs.

Vaccination of patients with tumour lysate-pulsed monocyte-derived mDCs

One week before vaccination, the patients were interviewed and medical histories taken, and the following baseline studies were carried out. A physical examination and complete blood work up consisting of differentiation, blood chemistry and serology, including assays for C-reactive protein and tumour markers lactate dehydrogenase (LDH) and 5-*S*-cysteinyldopa (5-SCD) were performed. Whole-body computed tomography (CT) and delayed-type hypersensitivity (DTH) skin testing was carried out. Antinuclear antibodies (ANA) titres were determined in all patients upon enrolment in the study and after receiving 10 vaccinations to detect any serological autoimmune reaction. Each week for 10 weeks the eligible patients were injected intradermally with tumour lysate-pulsed monocyte-derived mDCs (10^7 cells/injection) in close proximity to the cervical and inguinal lymph nodes. The clinical response was evaluated according to World Health Organization (WHO) criteria. Patients were deemed to have had a 'mixed response' if some of their tumours showed regression by > 25% of the pretreatment mass while others showed progression by > 25% of the pretreatment mass or new metastases appeared. Adverse events were evaluated by grading the toxicity according to the National Cancer Institute (NCI) Common Toxicity Criteria (CTC) guidelines version 2. Two patients with mixed responses were enrolled in an additional 20-vaccination protocol after obtaining signed informed consent and the recommendation of the institutional review board.

Concurrent administration of rhIL2 with DC-based tumour vaccine

rhIL2 (Imunace, 350 000 IU/vial, Shionogi Pharmacy, Osaka, Japan) was administered subcutaneously into the forearm three times a week. The dose of rhIL2 was generally 700 000 IU/day, though it had to be reduced in one patient because of side effects (mainly fever lower than 38°C and eosinophilia).

DTH skin testing

DTH skin tests were performed 4 days before the first vaccination and then 5 and 10 weeks after it. Irradiated (150 Gy from a ^{137}Cs source) autologous melanoma cells (10^6 cells) or their lysates were injected intradermally into the patients' forearm. In addition, irradiated autologous PBMCs (10^6 cells) and their lysates were used as a negative control. Forty-eight hours after each injection, the diameter of the erythema and the induration were measured. Erythemas < 10 mm in diameter were defined as negative (−), those 10 mm in diameter were defined as neutral (±), and those > 10 mm in diameter were defined as positive (+). Erythemas > 10 mm with induration were defined as strongly positive (++).

Migration assay

In order to investigate the chemotaxic migration of iDCs and mDCs toward RANTES and MIP-3β, polycarbonate membrane filters (pore size 8.0 μm) were precoated with 5 μg of gelatin. The lower chambers contained 600 μl of medium with or without 100 ng/ml RANTES and MIP-3β. The upper chamber contained 100 μl of cell suspension (10^6 cells). The chambers were incubated at 37°C for 4 h. After staining with haematoxylin and eosin, the number of migrated cells per HPF was counted (magnification ×400).

Allogeneic MLR

T-cells were isolated to > 98% purity from PBMCs as described previously [13]. Allogeneic T-cells (10^5 cells) were co-cultured with irradiated (15 Gy from a ^{137}Cs source) DCs (10^4 cells) in 96-well flat-bottom microplates (Coster, Cambridge, Massachusetts, USA). Thymidine incorporation was measured on day 5 following an 18 h pulse with [^{3}H]thymidine (1 μCi/well, specific activity 5 Ci/mmol, Amersham Life Science, Little Chalfont, Buckinghamshire, UK).

Cytotoxicity assay

PBMCs were obtained from four melanoma patients (patients 5, 6, 8 and 9) 10 weeks after the first vaccination and cultured for 4 days in medium containing 100 U/ml rhIL2 plus 100 μg/ml autologous tumour lysates. CD2+ lymphocytes were then positively selected from IL2-activated tumour lysate-pulsed PBMCs using anti-CD2 MAb conjugated immunomagnetic beads. CD2+ cells (10^4 to 5×10^5 cells) were co-cultured for 4 h in 96-well round-bottom plates with autologous or allogeneic melanoma cells (10^4 cells) labelled with $Na_2{}^{51}CrO_4$ (100 μCi/10^6 cells, NEN Life Science Products, Boston, Massachusetts, USA) at effector cell-to-target cell ratios ranging from 25 to 200 (Coster). The supernatants were subsequently harvested, the radioactivity counted, and the percentage of specific lysis calculated [13]. The counts per minute (c.p.m.) for spontaneous release were < 20% of the total release c.p.m.

Statistical analysis

The statistical significance of differences between the two groups was evaluated using the non-parametric Mann–Whitney *U*-test.

Results

Patient characteristics and clinical outcome

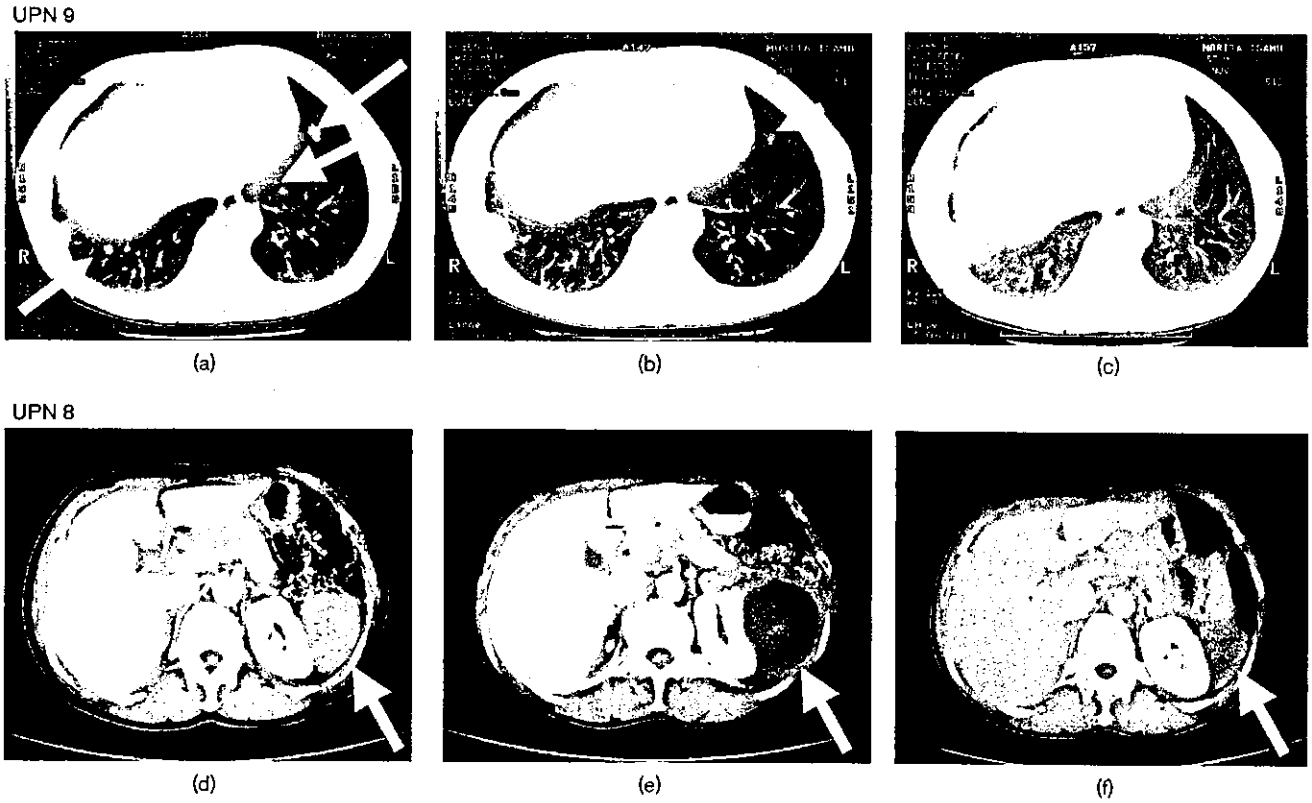
Ten stage IV malignant melanoma patients whose clinical status varied widely were enrolled into our study; their previous therapies and clinical responses are summarized in Table 1. Following the 10 week therapeutic protocol, analysis of total body CT images revealed a reduction in tumour metastasis in two patients (patients 8 and 9). Figure 1a–c shows the disappearance of multiple lung metastases in patient 9, while Figure 1d–f shows necrosis and regression of a metastatic lesion in patient 8. Necrosis of numerous metastatic lesions was observed during treatment in both patients 8 and 9. Figure 2 shows a pathological specimen from a metastatic skin lesion after treatment in patient 8; note the prominent central necrosis. Necrosis of metastatic lesions was accompanied by local pain, elevated C-reactive protein and sometimes fever; necrosis of skin lesions was also accompanied by local erythema. However, because other metastatic lesions in patients 8 and 9 showed increased tumour mass, the final response of these patients was deemed to be mixed. The clinical response of patient 6 was stable disease, while that of the remaining seven patients was progressive disease, although the rate of increase in total tumour mass declined slightly following treatment in patient 5 (data not shown).

Adverse events are summarized in Table 2. Mild fever (38°C) was occasionally seen for 1–2 days, or a transient erythema and induration occurred around the vaccination sites. We observed no clinical signs of autoimmune disease, and the antinuclear antibody titres were not elevated after therapy. There was no sign of NCI-CTC grade 3–4 toxicity following DC-based vaccination. We therefore conclude that our phase I immunotherapy protocol using autologous, tumour lysate-pulsed, monocyte-derived mDCs plus rhIL2 can be applied repeatedly without substantial side effects.

DTH reaction of melanoma patients

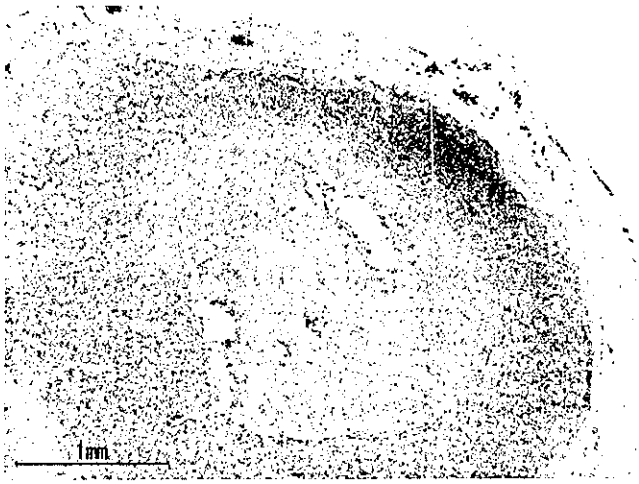
To evaluate tumour-specific immunity, we examined the DTH responses toward irradiated autologous melanoma cells and their lysates (Table 1). Before treatment, there was little or no DTH response against these samples, except in patient 7. By contrast, 5 and 10 weeks after the first vaccination, DTH responses against irradiated autologous melanoma cells and their

Fig. 1



CT of the lungs at about the same level in patients 9 (a–c) and 8 (d–f) obtained before (a,d), during (b,e) and after (c,f) treatment. Arrows indicate metastases. The disappearance of multiple lung metastases in patient 9 (a–c) and the regression of retroperitoneal metastasis in patient 8 (d–f) can be seen.

Fig. 2



Resected specimen of a metastatic skin lesion from patient 8; note the central necrosis. Scale bar: 1 mm.

lysates were detected in five of the patients (patients 2, 3, 4, 5 and 6). No responses to irradiated autologous PBMCs or their lysates were detected (data not shown).

Quality control of administered patient-derived DCs and comparison with those from healthy volunteers

To compare the biological properties of monocyte-derived DCs from melanoma patients and healthy volunteers, we first examined the cell surface expression of DC-family markers (CD1a and CD11c), co-stimulatory molecules (CD40, CD80 and CD86) and HLA-DR (Fig. 3). Flow cytometric analysis revealed levels of CD1a expression on iDCs from melanoma patients that were significantly lower than on those from healthy donors (Fig. 3a, $P < 0.001$). Expression of the other molecules tested was similar in the two groups of iDCs (Fig. 3b–g). TNFα-stimulated mDCs from melanoma patients and healthy volunteers expressed similar levels of CD83, which is known to be a maturation marker for a family of DCs [6] (Fig. 3e). On the other hand, expression of CD1a, CD40, CD80, CD86 and HLA-DR was upregulated on mDCs from healthy volunteers, but not on those from melanoma patients (Fig. 3a, c, d, f and g); indeed, cell surface expression of CD86 and HLA-DR was significantly downregulated ($P < 0.001$) on melanoma patient-derived mDCs (Fig. 3f,g).

To assess induction of DC endocytotic activity by TAA/tumour lysates from melanoma patients and healthy volunteers, mannose-receptor-mediated endocytosis of FITC-DX and macropinocytosis of LY via cytoskeleton-dependent fluid-phase endocytosis were evaluated (Fig. 4). The amounts of FITC-DX (Fig. 4a) and LY (Fig. 4b) internalized by iDCs from melanoma patients tended to be lower than the amounts internalized by iDCs from healthy volunteers, though the effect was only significant for LY. The capacity to internalize FITC-DX and LY was downregulated in both types of mDC.

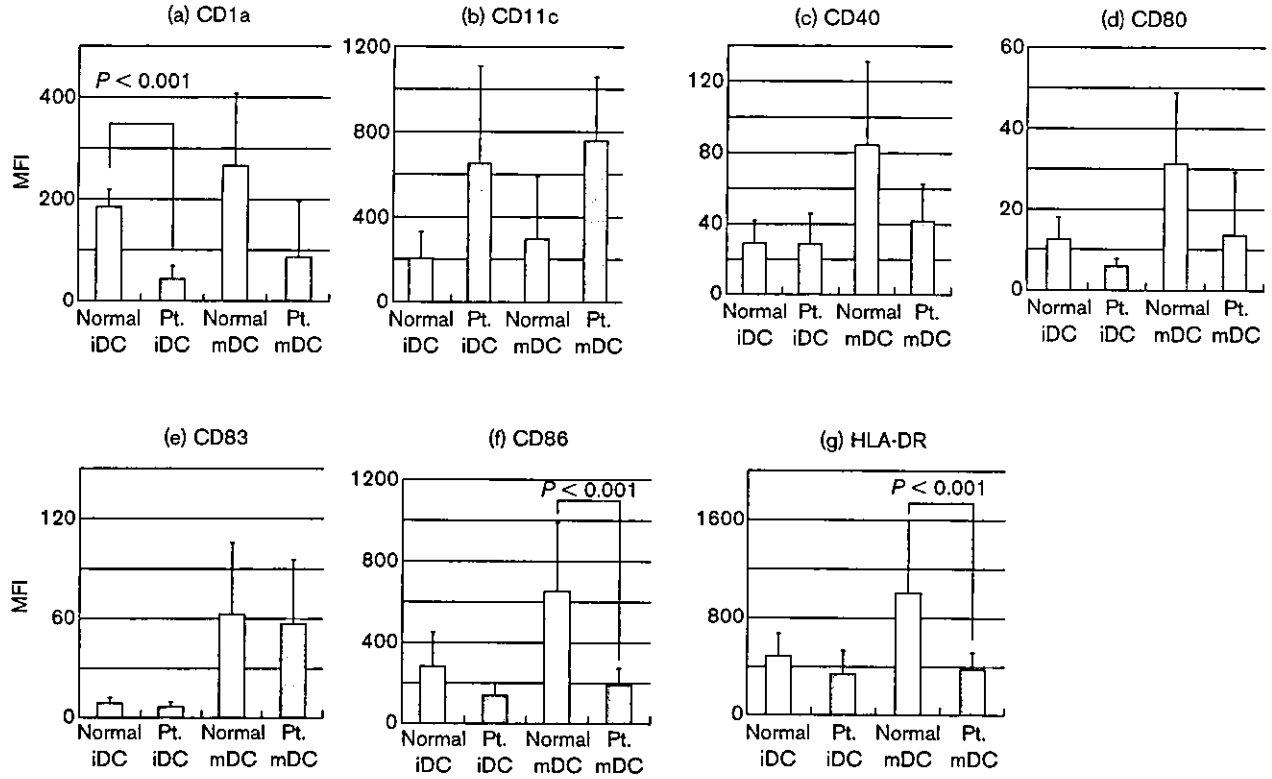
iDCs are known to migrate toward sources of inflammatory chemokines, while mDCs migrate toward sources of homeostatic chemokines [2,3,11]. To compare the responsiveness to chemokines of DCs from melanoma patients and healthy volunteers, we examined the chemotactic migration induced by RANTES and MIP-3β. We found that iDCs from melanoma patients responded less to RANTES than those from healthy volunteers (Fig. 5a, $P < 0.05$), and that RANTES-induced chemotaxis was diminished in both types of mDC (Fig. 5b). mDCs from both melanoma patients and healthy volunteers responded to MIP-3β, though

Table 2 Adverse events accompanying DC vaccination (NCI-CTC grading)

	Patient no.									
	1	2	3	4	5	6	7	8	9	10
Blood/bone marrow										
Platelets	0[a]	0	0	0	0	0	0	0	0	0
Constitutional symptoms										
Fever	0	0	0	0	0	0	0	0	0	0
Weight gain	0	0	0	0	0	0	0	0	0	0
Weight loss	0	0	0	0	0	0	0	0	0	0
Dermatology/skin										
Injection site reaction	1	0	0	0	0	0	1	0	0	0
Tumour site reaction concurrent with tumour regression	0	0	0	0	0	0	0	1	1	0
Autoimmune reaction										
ANA before treatment[b]	< 40×	< 40×	< 40×	< 40×	< 40×	< 40×	< 40×	< 40×	< 40×	< 40×
ANA after completion of treatment[b]	< 40×	< 40×	< 40×	< 40×	< 40×	< 40×	< 40×	< 40×	< 40×	< 40×

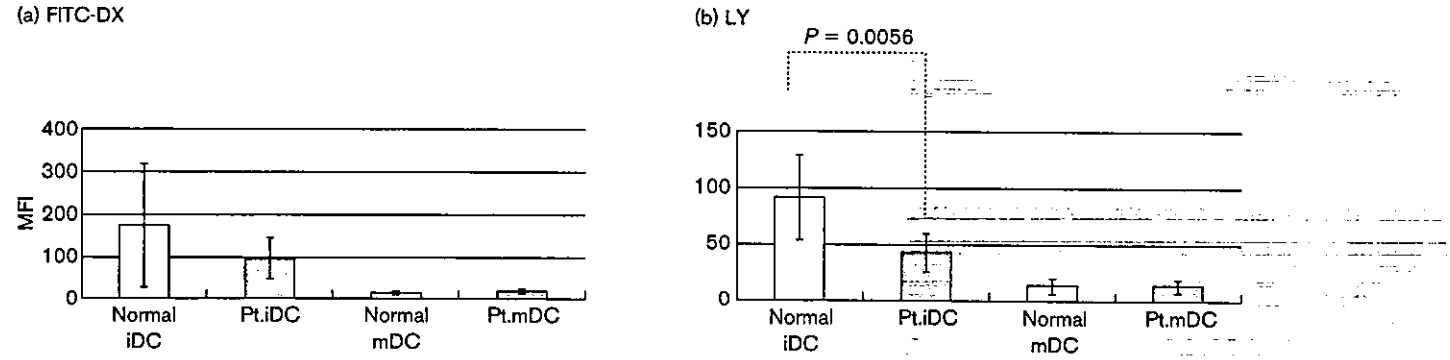
ANA, antinuclear antibody titre.
[a]This case exhibited lower platelet counts (grade 2–3) due to complicated disseminated intravascular coagulation; consequently this adverse event was considered to be unrelated to the DC vaccination.
[b]Fold of titre.

Fig. 3



Immunophenotyping of iDCs and normal mDCs derived from patients and healthy volunteers. Cell surface expression of CD1a (a), CD11c (b), CD40 (c), CD80 (d), CD83 (e), CD86 (f) and HLA-DR (g) was analysed by flow cytometry. The bars depict the mean ± SD for iDCs and normal mDCs from healthy volunteer donors (Normal; $n = 12$; white bars) and melanoma patients (Pt.; $n = 10$; grey bars). Values were compared using the Mann–Whitney's *U*-test. MFI, mean fluorescence intensity.

Fig. 4

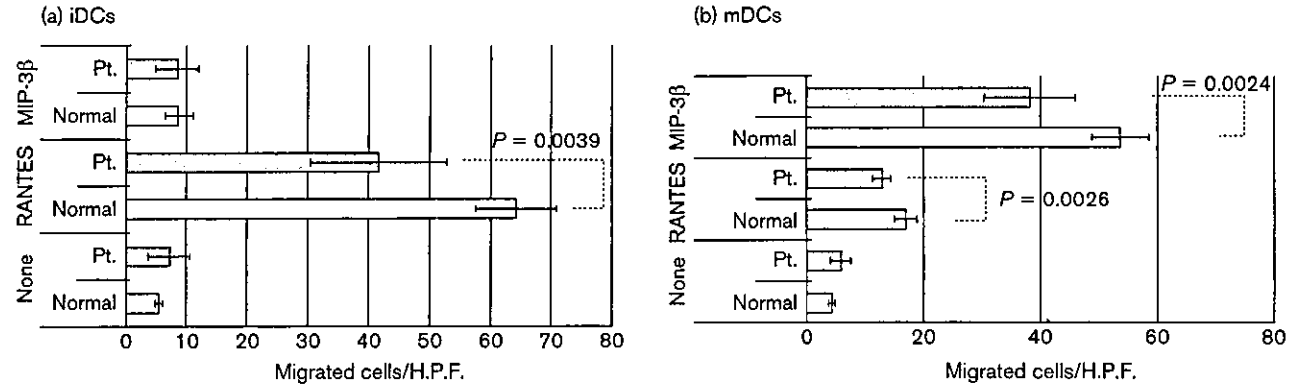


Flow cytometric analysis of the antigen uptake capacities of iDCs and mDCs. The bars depict the mean ± SD amount of FITC-DX (a) and LY (b) internalized at 37°C over a period of 1 h by iDCs and mDCs from healthy volunteers (Normal, white bars) and melanoma patients (Pt., grey bars). MFI, mean fluorescence intensity.

patient-derived mDCs were less responsive than those derived from healthy volunteers (Fig. 5b, $P < 0.05$ for both RANTES and MIP-3β).

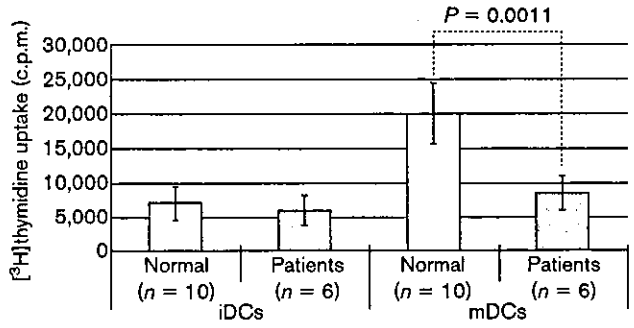
We also found that iDCs from the patients and healthy volunteers exhibited similar capacities to stimulate allogeneic T-cells (Fig. 6). This capacity was not enhanced in mDCs from melanoma patients, but was enhanced in mDCs from healthy volunteers ($P < 0.05$). It thus appears that changes in the expression of MHC pro-

Fig. 5



Chemotaxic migration of iDCs (a) and mDCs (b) from melanoma patients (grey bars) and healthy volunteer donors (white bars) toward RANTES and MIP-3β. Polycarbonate membrane filters (pore size 8.0 μm) were precoated with 5 μg of gelatin. The lower chambers contained 600 μl of medium with or without 100 ng/ml RANTES and MIP-3β. The upper chamber contained 100 μl of cell suspension (10^6 cells), which were incubated at 37°C for 4 h. After staining with haematoxylin and eosin, the numbers of migrated cells per HPF were counted at magnification ×400.

Fig. 6



Stimulation of allogeneic T-cells by DCs from melanoma patients and normal healthy volunteers. T-cells isolated from peripheral blood were cultured for 5 days with irradiated DCs (10^4 cells), after which [^{3}H]thymidine incorporation was determined.

duct/co-stimulatory molecules paralleled the ability of these cells to stimulate allogeneic T-cells.

In vitro cytolytic activities of IL2-activated CD2+ lymphocytes

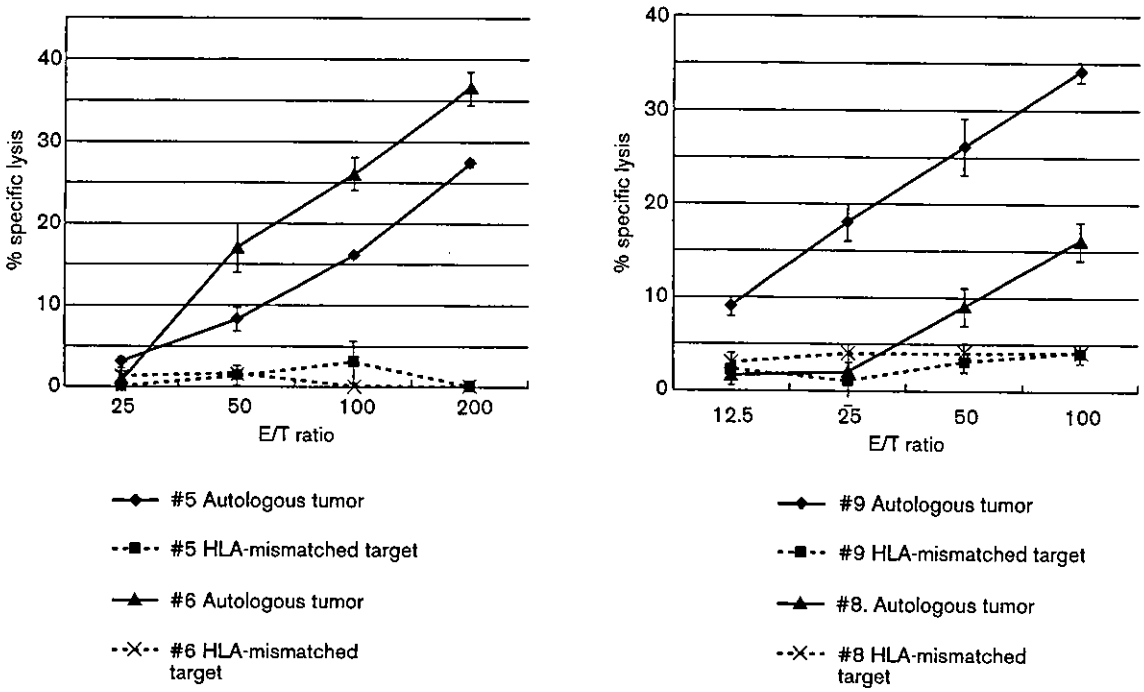
We examined the cytotoxic activity of PBMCs against autologous and allogeneic melanoma cells derived from four melanoma patients (patients 5, 6, 8 and 9). When PBMCs were stimulated with tumour lysate plus rhIL2 *in vitro*, we detected cytolytic activity by CD2+ lymphocytes against autologous melanoma cells, but not against HLA-mismatched allogeneic melanoma cells (Fig. 7). In contrast, no such activity was detected with CD2+ lymphocytes obtained from melanoma patients before treatment (data not shown).

Discussion

We have described a pilot study in which an immunotherapy protocol involving administration of a DC-based vaccine plus low dose rhIL2 was used to treat stage IV malignant melanoma patients unresponsive to other therapies. Our protocol entailed several modifications of a previously described immunotherapy with a DC-based vaccine [7]. First, we carried out a single 15 l leukapheresis on each melanoma patient to obtain $> 10^8$ monocyte-derived DCs, which enabled us to vaccinate each patient 10 times (10^7 tumour lysate-pulsed mDCs per injection). Thus, leukapheresis provided us with a means to obtain large numbers of DC-based cells without requiring patients to undergo frequent blood collection. Second, we used mDCs, rather than iDCs, as a source of DC-based vaccine because (i) mDCs show superior ability to induce Th1 and cytotoxic T-lymphocyte responses [1]; (ii) mDCs are more resistant to the immunosuppressive effect of IL10 than iDCs [14]; and (iii) mDCs more efficiently home into and accumulate within T-cell-dependent areas of secondary lymphoid tissues than iDCs [1]. Third, we combined DC-based vaccination with low dose administration of rhIL2. Shimizu *et al.* [15] reported that administration of IL2 enhances the therapeutic efficacy of DC-based vaccines in murine experimental models [15]; it is also well known that IL2 acts not only as a growth factor for lymphocytes but also enhances their cytolytic activities. Moreover, DCs directly activate natural killer (NK) [16] and natural killer T (NKT) [17] cells, and rhIL2 may potentiate this effect.

Our data show that the side effects of this approach were negligible, though most of the patients remained unresponsive to therapy. However, two patients did show a mixed response, and others showed enhancement of *in vivo* DTH responses against irradiated autologous melanoma cells and their lysates (Table 1) as well as cytolytic responses of *in vitro*-activated CD2+ lymphocytes against autologous melanoma cells (Fig.

Fig. 7



Cytolytic activity of *in vitro*-activated, patient-derived CD2+ lymphocytes against autologous or allogeneic melanoma cells. Lysis was measured in terms of the percentage ^{51}Cr release as described in Materials and methods.

7). Moreover, our finding that patient-derived melanoma cells are a heterogeneous population in terms of their expression of MHC products and the CD86 co-stimulatory molecule suggests that tumour lysate-pulsed DCs and rhIL2 synergistically activated lymphocytes to kill melanoma cells through both MHC-dependent and MHC-independent mechanisms. It therefore appears that immunotherapy with a DC-based vaccine and rhIL2 has the potential to give protective immunity against melanoma cells in stage IV with no NCI-CTC grade 3–4 side effects. Taken together, these findings provide a clinical safety rationale for the use of a DC-based vaccine in patients with advanced melanoma and indicate that further research into improving the efficacy of this approach is clearly warranted.

We were also interested in whether our therapeutic protocol could induce tumour-specific immunity in melanoma patients. Our data showed enhanced DTH responses against melanoma cells and enhanced cytolytic responses by *in vitro*-activated CD2+ lymphocytes against autologous melanoma cells in some patients. The diminished lung metastasis observed in patient 9 and the central necrosis observed in both patients 8 and 9 appear to reflect the antitumour immunity induced by DC therapy, though the precise cause of these effects is not yet certain. Nestle *et al.* [7] previously reported an association between the clinical response and the DTH response in advanced melanoma patients receiving DC-based vaccine. However, our findings and those of Thurner *et al.* [8] suggest that, although we were able to induce a degree of protective immunity against melanoma cells (e.g. DTH responses), there was not necessarily an association with clinical outcome. This discrepancy probably reflects differences in the clinical status of the patients and the experimental designs, which suggest that a balance between the degree of protective immunity induced and the patient status (e.g. the tumour burden) may determine the clinical outcome in malignant melanoma patients given this type of immunotherapy.

Central necrosis of metastatic tumours was a prominent feature of the antitumour response observed in our study. It is now well established that tumour angiogenesis is regulated by the vascular endothelial growth factor (VEGF) family of cytokines [18–20], and that DCs produce large amounts of interferon-γ (IFNγ) in response to IL12 stimulation [12]. The observed necrosis may therefore reflect inhibition of the VEGF cascade, and thus tumour angiogenesis, by IFNγ released from administered DCs or from activated Th1/NK cells.

Why the treatments were ineffective in most patients remains unclear. It may be that melanoma cells escape host protective immunity during the course of treatment. For example, melanoma cells reportedly produce large amounts of IL10 and transforming growth factor-β (TGFβ), which suppress host anticancer immunity [21–23]. Yue *et al.* [21] described the transcription and translation of IL10 and IL10 receptor (IL10R) in patient-derived melanoma cells, as well as decreased cell surface expression of HLA class I, class II and intercellular adhesion molecule-1 (ICAM-1), which reciprocally regulates IL10R expression [21]. In addition, we have previously shown that IL10 interferes with TNFα-induced maturation of iDCs [10], and expression of HLA-DR was diminished on mDCs from melanoma patients in the present study. Taken together, these findings strongly suggest an IL10-mediated escape of melanoma cells from host immune surveillance. Steinbrink *et al.* [22] described a melanoma antigen-specific anergy caused by treating DCs with IL10, and Enk *et al.* [23] suggested that DCs themselves mediate tumour-induced tolerance in metastatic melanoma. This means that IL10 and TGFβ1 may induce the dysfunctional immune properties seen in the patient-derived DCs examined in the present study. The use of HLA-matched unrelated donor-derived monocytes as sources of DCs, or gene transduction of appropriate immunostimulatory molecules into autologous DCs and tumour cells, may enable us to overcome the impaired host antitumour immunity.

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17β-Estradiol inhibits cardiac fibroblast growth through both subtypes of estrogen receptor

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Abstract

The effect of 17β-estradiol (E2) on the proliferation of cardiac fibroblasts (CFs) remains controversial. This study investigated which subtype of estrogen receptor (ER), ERα or ERβ, mediated the effect of E2 on CF growth by the gain of function analysis using an adenovirus vector. One hundred nanomoles per liter of E2 attenuated DNA synthesis by up to 10%, and transactivated the estrogen-responsive element determined by luciferase assay in rat neonatal CFs. We constructed replication-deficient adenoviruses bearing the coding region of human ERα, ERβ, or the dominant-negative form of ERβ (designated AxCAERα, AxCAERβ, and AxCADNERβ, respectively). When CFs were infected with AxCAERα or AxCAERβ at multiplicity of infection of 20 or higher, DNA synthesis was decreased by 50% in response to E2 and the effect was abolished by co-infection with AxCADNERβ. Similarly, transcriptional activity of ER in CFs infected with AxCAERα or AxCAERβ was markedly enhanced and co-infection with AxCADNERβ abolished the effects. These results suggest that E2 inhibits CF growth and that both ER subtypes mediate the effect comparably and redundantly.

Keywords: Cardiac remodeling; Cardiac fibroblast; Hormones; Receptor; Adenovirus

Structural remodeling of the ventricular wall takes place in several cardiac disorders including acute myocardial infarction, cardiomyopathy, and hypertensive heart disease. Histopathologically, it is characterized by a structural rearrangement of components of the normal chamber wall that involves cardiomyocyte hypertrophy, proliferation of cardiac fibroblast (CFs), fibrosis, and cell death [1]. In the adult heart, CFs substantially constitute the non-myocyte cells [2] and contribute to cardiac remodeling by undergoing proliferation, depositing extracellular matrix proteins which are mainly produced by CFs in the myocardium, and eventually replacing myocytes with fibrotic scar tissue. CFs also produce matrix metalloproteinases, growth factors, and cytokines, all of which are involved in the maintenance of myocardial structure, and in diseased hearts play pivotal roles in remodeling [3]. Recent studies have shown that the interactions between CFs and cardiomyocytes are essential for the progression of cardiac remodeling [3]. Thus, it is clinically important to inhibit CF growth in the process of cardiac remodeling.

From several epidemiological studies, estrogen (E2) is thought to have a protective effect against left ventricular hypertrophy which is an important cardiovascular risk factor for morbidity and mortality [4–6]. Premenopausal women have a lower prevalence of left ventricular hypertrophy than their age-matched male counterparts [4]. Left ventricular mass is significantly greater in men than in women even after indexing for body surface area [5,6]. Experimental studies have shown cardioprotective roles of E2 [7–10], however, the direct effect of E2 on cardiac cell growth remains to be determined. Previous studies have demonstrated that the

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exogenous administration of E2 either decreased [7], increased [11], or had no effect on DNA synthesis in cultured CFs [12,13].

Most biological effects of E2 are mediated by the estrogen receptor (ER). ER has two subtypes, classical ERα and newly identified ERβ [14]. It is reported that both ER subtypes are expressed in CFs [13,15]. However, little is known about the involvement of ER in CF growth, although many transcriptional factors including nuclear receptors regulate the functions of CFs in the process of cardiac remodeling [3]. There is only one report showing that the inhibitory effect of E2 on CF growth is independent of ER [16]. Adenovirus-mediated gene transfer is a useful tool to clarify the precise role of a specific gene. We constructed replication-deficient adenovirus vectors carrying ERα, ERβ or dominant-negative form of ERβ. In this study, to determine the effect of E2 on CF growth and which ER subtype plays a pivotal role in the cell growth, we evaluated DNA synthesis in CFs overexpressing each ER subtype using adenovirus vector. Here we show that E2 attenuated DNA synthesis by up to 10% in rat neonatal CFs and that adenovirus-mediated overexpression of either of the ER subtypes in CFs augmented growth inhibition in a ligand-dependent manner.

Methods

Cell culture. Rat CFs were harvested from the heart of Wistar neonatal rats at birth, as previously reported by Zang et al. [17] Briefly, the hearts were removed from neonatal rats and minced with scissors until very small pieces were produced. The pellet of minced tissue was then resuspended in 1% collagenase and incubated at 37 °C for 2 h. Next, the tissue was resuspended in 0.25% trypsin and incubated at 37 °C for 2 h. The digested tissue was resuspended in Dulbecco's modified Eagle's medium (DMEM; Nikken Bio Medical Laboratory, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Intergen, Purchase, NY), 25 mM Hepes (pH 7.4), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Twenty-four hours later, the medium was aspirated and the fresh medium was added. CFs at 6–9 passages were used in the experiments. At the time of experiments, we used dextran-coated charcoal-stripped FBS (DCC-FBS) and phenol-red-free M199 medium to avoid contamination with steroids or estrogen receptor agonists.

Construction of adenovirus vectors carrying estrogen receptor subtypes and transfer into CFs. Replication-deficient adenovirus vectors carrying the CMV-IE enhancer, chicken β-actin promoter, and the coding region of human ERα, ERβ, or dominant-negative form of ERβ, were constructed by use of adenovirus expression vector kit (Takara Shuzo, Kyoto, Japan) as described before [18] and named AxCAERα, AxCAERβ, and AxCADNERβ, respectively. CFs were exposed to different multiplicities of infection (MOI) of either AxCAERα, AxCAERβ, AxCADNERβ, or a replication-deficient recombinant adenovirus carrying the *Escherichia coli* β-galactosidase gene (AxCALacZ) for 2 h in DMEM with 5% FBS. Then, the cells were rinsed with phosphate-buffered saline once and used for the experiments.

RNA isolation, reverse transcription polymerase chain reaction. Total RNA was prepared from CFs and rat ovary as positive control, using Isogen (Wako Pure Chemical Industries, Osaka, Japan). Then, 1 μg total RNA was reverse transcribed into cDNA and one-twentieth of the product was amplified for 35 cycles. Negative control reverse transcription polymerase chain reactions (RT-PCRs) were performed by omitting reverse transcriptase. The primer pairs used in PCR are: CTAAGAAGAATAGCCCCGCC (forward, +1126 to +1145) and CAGACCAGACCAATCATCAGG (reverse, +1402 to +1382) for rat ERα (GenBank Accession No. NM_012689), and CGACTGAGCAC AAGCCCAAATG (forward, +76 to +97) and ACGCCGTAA TGATACCCAGATG (reverse, +353 to +332) for rat ERβ (GenBank Accession No. AB012721).

Measurement of [^{3}H]thymidine incorporation. CFs seeded onto 24-well tissue culture plates were grown until 70–90% confluent and then made quiescent by culturing in phenol-red-free M199 medium (Gibco) for 24 h. Then, the cells were stimulated with 5% DCC-FBS in the presence of water-soluble 17β-estradiol (Sigma–Aldrich, Japan) for 24 h, followed by pulse-labeling with 1 μCi/ml [^{3}H]thymidine for 3 h. [^{3}H]Thymidine incorporated into DNA was determined as previously described [19].

Number of CFs. CFs were seeded onto six-well multiplates and cultured until a confluent state was obtained. After infection of CFs with adenovirus vectors, the medium was replaced with phenol-red-free M199 to arrest the growth. After 24 h, the medium was replaced again with phenol-red-free M199 containing 5% DCC-FBS with E2 or vehicle. After incubation for 48 h, the cells were trypsinized and suspended. Then the number of cells was determined using a Coulter Counter (model ZM, Coulter Electronics, Hialeah, FL).

Luciferase assays. CFs were transfected with ERE-TK-Luc reporter plasmid and pRL-SV40 control plasmid using FuGENE6 (Roche) for 24 h according to the manufacturer's instructions [20]. Then, CFs were incubated in phenol-red-free M199 medium with 1% DCC-FBS for 24 h and exposed to E2 for additional 24 h. We measured two kinds of luciferase activity using a dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol, and the ratio of firefly luciferase activity to that of *Renilla* luciferase in each sample was used as a measure of normalized luciferase activity [20].

Western blotting. After infection with adenovirus vector, cells were incubated with serum-free M199 medium for 24 h to detect ER subtypes. Cells were washed quickly with phosphate-buffered saline twice and lysed in RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors cocktail; Complete, Mini; Roche). The samples were separated on 12% SDS–PAGE, electroblotted onto nitrocellulose membrane, and immunoblotted with anti-ERα polyclonal antibody (H-184; Santa Cruz, 1:1000 dilution), anti-ERβ monoclonal antibody (CWK-F12, kindly provided by Dr. Benita S. Katzenellenbogen, thanks and details are given in Acknowledgements, 1:1000 dilution). Antibody was detected with a horseradish peroxidase-linked secondary antibody using an enhanced chemiluminescence system (Amersham Life Science).

Statistical analysis. The dose–response effect of E2 or ER overexpression on DNA synthesis in CFs was analyzed using one-way factor ANOVA. If a statistically significant effect was found, Newman–Keuls test was performed to isolate the difference between the groups. A value of $P < 0.05$ was considered statistically significant. All data in the text and figures are expressed as means ± SE.

Results

Endogenous expression of ER subtypes and the effect of E2 on CF growth

To investigate the endogenous expression of ER in rat CFs, RT-PCR amplification was performed. Both rat ERα and ERβ were expressed in CFs (Fig. 1A). At physiological concentrations, E2 inhibited the proliferation of CFs dose-dependently by up to 10% (Fig. 1B).

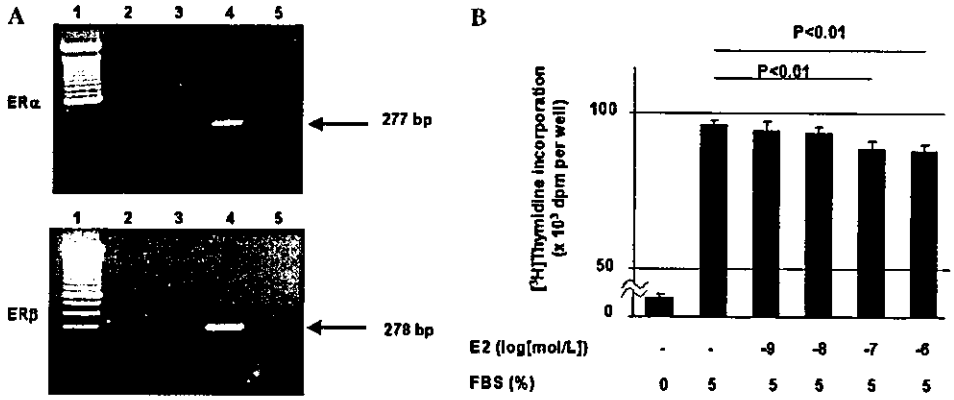

Fig. 1. ER gene expression and the effect of E2 on rat CF proliferation. (A) RT-PCR was performed using the cDNA of rat ovary as a positive control with (lane 4) or without (lane 5) reverse transcriptase and using the cDNA of rat CFs with (lane 2) or without (lane 3) reverse transcriptase. Lane 1 shows the molecular weight marker. B) Serum-starved CFs were stimulated with 5% DCC-FBS in the absence or presence of 10–1000 nmol/L 17β-estradiol for 24 h. [³H]Thymidine incorporation into DNA was determined by pulse-labeling for the last 3 h of incubation. Results are shown as means ± SE ($n = 3$). Similar results were obtained in three independent experiments.

Expression of ER subtype in CFs by adenovirus-mediated transfer of the ER subtype genes

Expression of the ERα and ERβ protein was confirmed by Western blot analysis (Fig. 2A). Although both ER subtypes were detected by RT-PCR, the protein expression was undetectable in non-transfected CFs; the bands corresponding to ERα (65 kDa) or ERβ (55 kDa) were seen in CFs infected with AxCAERα, or with AxCAERβ, respectively (Fig. 2A), and also in MCF-7 cells or rat ovary which were used as positive controls (data not shown). The protein expression was increased by overexpression MOI-dependently. We also checked the protein level of both ER subtypes in non-infected cells after the addition of E2. However, E2 did not induce the protein of either ER subtype in our experimental conditions (data not shown).

Effect of adenovirus-mediated transfer of the ER subtype genes on CF growth

When AxCALacZ was introduced into CFs at more than 60 MOI, DNA synthesis reduced in a MOI-dependent manner in the absence of E2 (data not shown). Therefore, we examined DNA synthesis at 60 MOI or less to avoid the influence of adenovirus itself on DNA synthesis. CFs infected with AxCALacZ showed no additional decrease in DNA synthesis in response to E2 (Fig. 2B). In contrast, when CFs were infected with AxCAERα or AxCAERβ at more than 10 MOI, DNA synthesis was significantly inhibited in a MOI-dependent manner in response to E2 to grossly similar extent. To confirm this, the cell number was counted in the presence or absence of E2. Comparable to the thymidine incorporation assay, overexpression of either ERα or ERβ enhanced the inhibitory effect of E2 on CF growth (Fig. 2C). Moreover, in CFs infected with either AxCAERα or AxCAERβ at 20 MOI, E2 decreased DNA synthesis in a concentration-dependent manner at 10^{-11}–10^{-6} mol/L (Fig. 3). Taking these results together, the effects of AxCAERα and AxCAERβ seemed comparable. To examine whether the effect of ER transfer is truly ER subtype dependent, we investigated DNA synthesis in CFs co-infected with AxCAERα or AxCAERβ and AxCAERDNβ. The reduction of DNA synthesis in CFs infected with AxCAERα or AxCAERβ alone at 20 MOI was abolished by co-infection with AxCAERDNβ (Fig. 4).

Transcriptional activity of ERE in CFs infected with ER genes

We examined the transcriptional activity of ER by luciferase activity of the ERE reporter plasmid. In non-infected CFs, 100 nmol/L E2 augmented the luciferase activity of ERE by approximately 1.3-fold compared to vehicle ($p = 0.02$) (Fig. 5). CFs infected with AxCAERα or AxCAERβ at 20 MOI showed a strong increase in transcriptional activity in the presence of E2; 3.3-fold increase with AxCAERα and 3.9-fold increase with AxCAERβ in response to E2. This increase was completely abolished by co-infection with AxCADNERβ.

Discussion

Conflicting results have been reported concerning the effect of E2 on CF growth. One group demonstrated that CF growth was not affected by E2 [12]. Two groups showed that E2 inhibited CF growth [7,13], whereas another has shown that E2 enhanced CF growth through mitogen-activated protein kinase-dependent pathway [11]. Thus, the effect of estrogen on CF growth remained to be addressed. In this study, E2 inhibited DNA synthesis in CFs by up to 10%, and this inhibition was augmented by overexpression of either of ER subtypes,

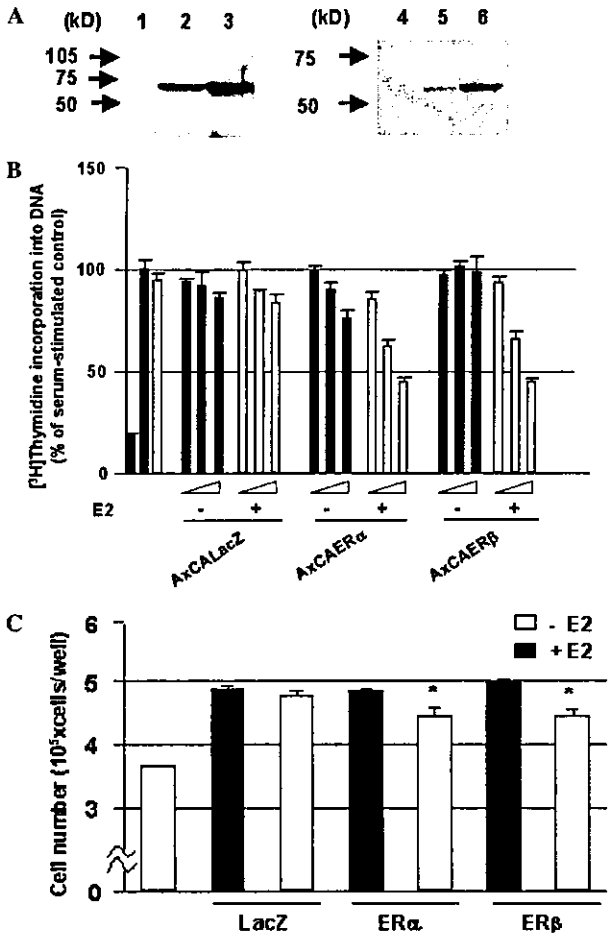

Fig. 2. Induction of ER protein and inhibition of CF growth by adenovirus-mediated transfer of ER genes. (A) CFs were infected without (lanes 1 and 4), or with 10 and 100 MOI of AxCAERα (lanes 2 and 3, respectively) or 10 and 100 MOI of AxCAERβ (lanes 5 and 6, respectively). Western blot analysis was performed with 40 μg of protein per lane by using an anti-ERα polyclonal antibody (left panel) or anti-ERβ monoclonal antibody (right panel). CFs seeded onto a 24-well plate (B) or 6-well plate (C) were exposed to DMEM containing either AxCALacZ, AxCAERα, or AxCAERβ (1, 10, and 30 MOI (B), respectively, from left to right, or 30 MOI (C)) for 2 h and serum-deprived for 24 h. [^{3}H]Thymidine incorporation into DNA (B) was determined at 24 h after the stimulation with 5% DCC-FBS in the presence or absence of 100 nmol/L E2 and presented as a percentage of the serum-stimulated control. The left-sided 3 lines indicate non-infected CFs with serum-free medium, 5% DCC-FBS in the absence of E2, and 5% DCC-FBS in the presence of 100 nmol/L E2, respectively (B). Cell numbers were counted after 48 h of stimulation with 5%DCC-FBS in the presence or absence of 100 nmol/L E2 (C). The left-sided line indicates non-infected CFs before the stimulation. $^*P < 0.01$ vs CFs without E2. Results are shown as means ± SE ($n = 3$) (B,C). Similar results were obtained in three independent experiments.

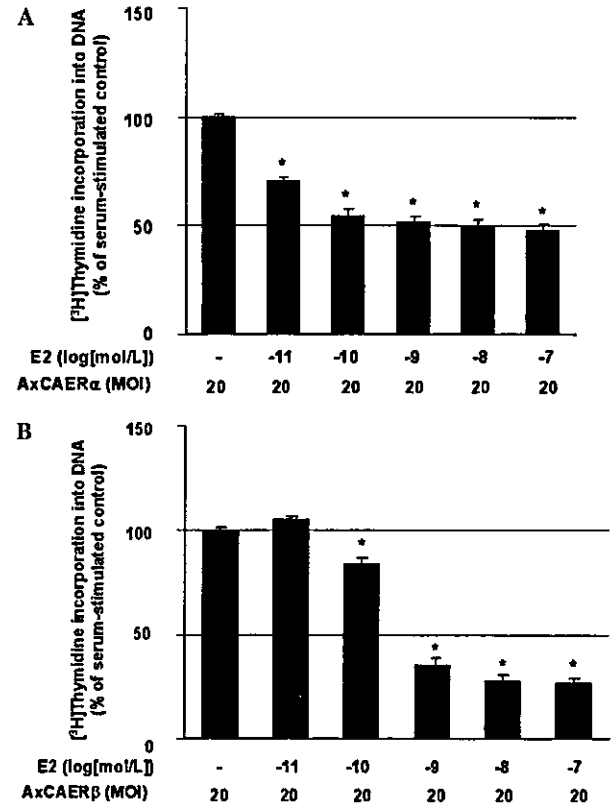

Fig. 3. Dose–response effect of E2 on DNA synthesis in CFs overexpressing ER subtypes. CFs seeded onto a 24-well plate were exposed to DMEM containing 10 MOI of AxCAERα (A) or AxCAERβ (B) for 2 h, and were serum-deprived for 24 h. [^{3}H]Thymidine incorporation into DNA was determined at 24 h after the stimulation with 5% DCC-FBS in the absence or presence of the indicated concentrations of E2 and presented as a percentage of CFs without E2. $^*P < 0.01$ vs CF without E2. Results are shown as means ± SE ($n = 4$). Similar results were obtained in three independent experiments.

indicating that both ER subtypes work to inhibit CF growth in a redundant fashion.

Both ER subtypes are expressed in cardiac myocytes and CF as shown by Western blotting [13,15,21], and are transcriptionally active [15], suggesting that ER subtypes play a role in cardiac cells. Moreover, it is reported that the expression of ER subtypes in cardiac cells was regulated by physiological or pathophysiological stimuli such as E2 [15] and hypoxia [22]. Protein levels of both ER subtypes were increased in CFs and cardiac myocytes in response to E2 [15]. Under hypoxic condition, the protein level of ERβ but not of ERα was upregulated while the presence of E2 decreased the level of ERβ protein in CFs [22]. Modulation of ER subtype expression by E2 was not confirmed in the present study (data not shown) presumably because the expression in non-transfected CFs was too low to detect by Western blotting. Changes of ER expression in cardiac cells associated with cardiovascular disease are currently unknown. However, the gain-of-function analysis implies the physiological relevance by mimicking the conditions of the previous reports [13,15,21,22]. Another rationale in using the overexpression system was to compare the effects on CF growth between ER subtypes. Because adenovirus vectors successfully induced ER subtypes to a similar extent, we could interpret the results clearly.

Several reports have examined the role of ER subtypes in proliferation using the gene transfer techniques into cell lines [23–26]. Cheng and Malayer [26] have reported that overexpression of ERα in an ER-negative

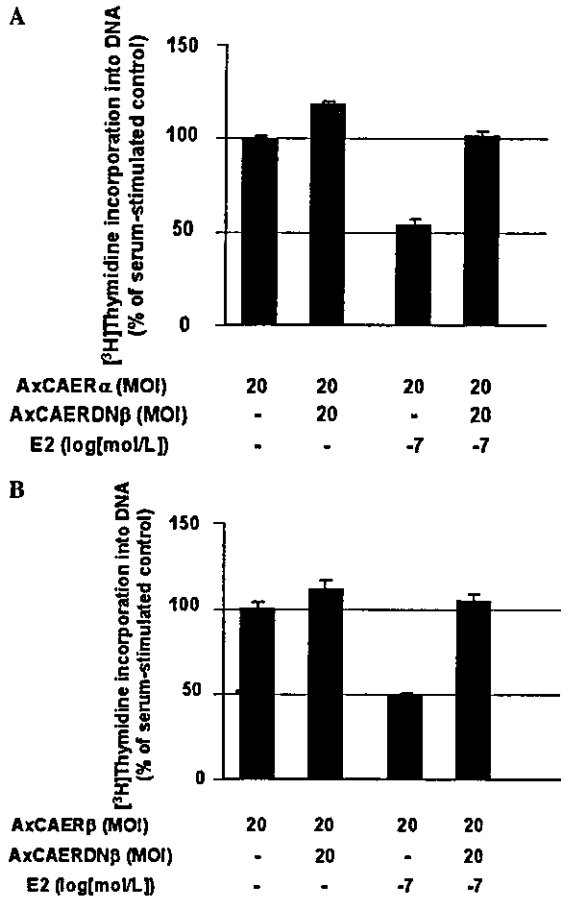

Fig. 4. The effect of dominant negative ER on ER subtype overexpression in CFs. CFs seeded onto a 24-well plate were exposed to DMEM containing 20 MOI of AxCAERα (A) or AxCAERβ (B) and the indicated MOI of AxCADNERβ. After infection, CFs were serum-deprived for 24 h. [³H]Thymidine incorporation into DNA was determined at 24 h after the stimulation with 5% DCC-FBS in the absence or presence of 100 nmol/L of E2, and were presented as a percentage of CFs infected with AxCAERα alone (A) or AxCAERβ alone (B) without E2. Results are shown as means ± SE ($n = 3$). Similar results were obtained in three independent experiments.

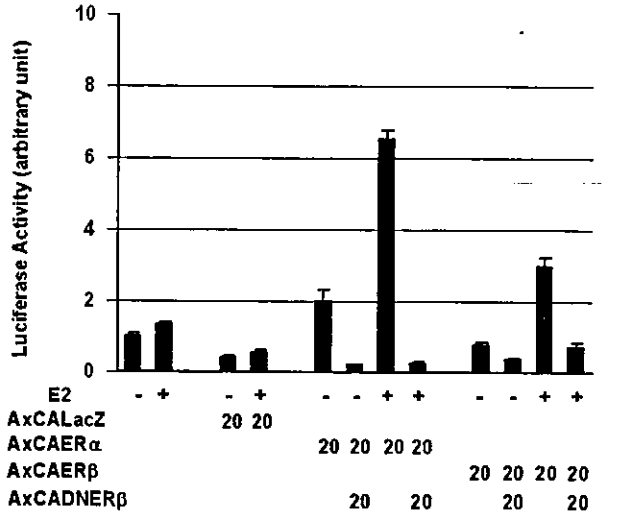

Fig. 5. The influence of ER overexpression on the promoter activity of ER responsive enhancer elements in CFs. CFs were infected with AxCALacZ, AxCAERα, AxCAERβ, or AxCADNERβ at the indicated MOI for 2 h, and transfected with the luciferase reporter plasmids containing ERE and the pRL-SV40 control plasmid. Twenty-four hours after transfection, the cells were treated with or without 100 nmol/L E2 for 24 h. Results are shown as means ± SE ($n = 3$). Similar results were obtained in three independent experiments.

rat fibroblast cell line, rat-1, resulted in an estrogen-dependent small (<10%) but significant increase in cell proliferation but overexpression of ERβ did not affect proliferation. In contrast, Lazennec et al. [24] have shown that overexpression of ERα in an ER-negative human breast cancer cell line, MDA-MB-231, led to a hormone-dependent inhibition of proliferation, whereas overexpression of ERβ caused a hormone-independent inhibition. Taken these results together with other reports examining the effect of ER overexpression in non-CF cells [23–25], the role of ER subtypes in cell proliferation may be different between cell types. This may also be the case with our results and the results in fibroblasts by Cheng and Malayer [26]. We used neonatal primary cultured CFs that expressed low levels of both ER subtypes, while Cheng et al. used a cell line derived from embryo fibroblasts that did not express ER.

The divergent roles of ER subtypes can be explained by the differential induction of estrogen response genes, the different interactions with promoter elements including AP-1 sites [27] and SP-1 sites [28] in an ERE-independent manner, or differential recruitment of transcriptional co-factors. In the present study, however, ERα and ERβ inhibited CF growth and transactivated the ERE similarly in response to E2. The only difference between ER subtypes observed in this study was that overexpression of ERα exerted the effects on cell growth and transcriptional activity ligand-independently (Figs. 2B and 5), although these effects were slight and might be non-specific. Accordingly, it is suggested that ERα and ERβ mediate the inhibitory effect of E2 on CF growth in a redundant or compensatory fashion as is the case with some gene superfamilies [29,30].

Our findings provide a mechanistic insight into the understanding of how E2 acts in CFs in the process of cardiac remodeling. Our data imply that the proliferation of CFs involved in cardiac hypertrophy and fibrosis can be inhibited by E2 as is shown in clinical and experimental settings [4–10], and that both ER subtypes expressed in CFs mediate the inhibitory effects of E2. Unfortunately, recent clinical trials [31,32] have failed to show beneficial effects of hormone replacement therapy on cardiovascular disease. Alternatively, specific ligands such as selective ER modulators [33] might exert beneficial clinical effects, particularly in combination with the gene transfer of ER subtypes, to inhibit cardiac remodeling. To test this possibility, in vivo experiments using ER overexpression and selective ER modulators should be performed in the future.

Acknowledgments

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Thyroglobulin-pulsed human monocyte-derived dendritic cells induce CD4$^+$ T cell activation

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Abstract. Although thyroglobulin (Tg) would be expected to act as a tumor-associated antigen that might be exploitable by immunotherapy against thyroid cancers, it remains unclear how to effectively enhance the immune response to Tg in human since it is a self-component glycoprotein. We therefore tested whether and how human peripheral blood (PB) monocyte-derived dendritic cells (DCs) pulsed with human (h)Tg would induce activation of hTg-specific T cells. We found that immature DCs (iDCs) exhibited a higher endocytic capacity for fluorescein isothiocyanate-conjugated hTg than did mature DCs (mDCs). Although freshly isolated T cells responded poorly to mDCs, hTg-primed T cells responded much more strongly to hTg pulsed mDCs, which selectively induced IFN-γ-secreting T cells. These results suggest that hTg-pulsed mDCs enhance the responses of Tg-specific T cells, raising the possibility that vaccination with hTg-pulsed mDCs may be an effective approach as immunotherapy to potentiate thyroid cancer specific therapy.

Introduction

Thyroid carcinomas, which are the most common malignancy in endocrine tumors, are histologically classified as papillary, follicular, medullary and anaplastic tumors. Although thyroid cancers are generally controllable by surgery or internal radiation therapy using ^{131}I, cancers often metastasize to the regional lymph nodes. Once thyroid cancer has metastasized to bone or the lung, particularly in older patients, it is known to be resistant to ^{131}I therapy or chemotherapy (1). The quality of life (QoL) of these patients is impaired, and their prognosis is not good (1). The transformation from papillary and follicular cancers to the much more virulent anaplastic form occurs in some patients, resulting in a very poor prognosis (the median survival is 2-6 months) (2). Therefore, a new therapy for metastatic or recurrenced thyroid cancers is now highly desirable to suppress the development of advanced or inoperable cancers.

Thyroglobulin (Tg) is a macromolecular glycoprotein consisting of two apparently identical polypeptide chains that together make up the mature 660 kDa, 19S dimer. This protein is secreted specifically by thyroid cells and provides a matrix for the synthesis of the thyroid hormones and a vehicle for their subsequent storage. All differentiated and some undifferentiated thyroid cancers also express Tg; in fact, blood Tg levels increase in thyroid cancer patients and are used clinically as a marker of the recurrence of a thyroid tumor or of the presence of residual tumors following surgical resection (3).

For the past several years, much attention has been paid to the development of cancer immunotherapies. Unfortunately, an earlier study has shown that immunotherapy involving vaccination with Tg elicits a poor clinical response in the thyroid cancer patients (4).

Recently, dendritic cells (DCs) are the most effective of the professional antigen (Ag)-presenting cells (APCs), which initiate and regulate immune responses (5), and the efficacy of immunotherapy using DCs pulsed with a tumor-associated antigen has been demonstrated in several phase I clinical studies (6,7). On the other hand, immunization with DCs pulsed with tissue specific Ag has been shown to cause several autoimmune diseases in animal models (8,9). From these points, thyroid cancer would seem to be a good candidate for immunotherapy using DCs. The thyroid gland is well known to be the target of autoimmune diseases such as Hashimoto's disease (10), in which thyroid tissues are destroyed by infiltrating T cells and replaced with fibrotic tissue (11,12). In an animal model of autoimmune thyroiditis, immunization with Tg plus adjuvant induces an experimental thyroiditis mediated through induction of Tg-specific T cells (13-15). The

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Key words: thyroglobulin, dendritic cell, CD4$^+$ T

immunization using DC pulsed with Tg (Tg-pulsed DCs) yields autoimmune thyroiditis (16). However, it can be tolerable unlike other organ specific antigens, because autoimmune thyroiditis is controllable by T3 supplement and normal thyroid gland is commonly resected in the operation of thyroid cancers. Tg, thus appears to have the potential to serve as a thyroid tumor-associated antigen for the induction of anti-thyroid, cancer-specific, T cell-mediated immunity. However it is still unclear whether Tg-pulsed DCs would elicit Tg-specific T cell responses in humans. Recently, mature DCs (mDCs) were established to induce T cell immunity, whereas immature DCs (iDCs) were involved in T cell tolerance (17).

In this study, therefore, we tested whether human monocyte-derived mDCs pulsed with hTg, even though it is a self antigen, induces activation of autologous hTg-specific T cells.

Materials and methods

Subjects. The study protocol was approved by the Ethics Committee of the Institute of Medical Science, University of Tokyo. Six healthy volunteers enrolled in this study. Before their enrollment, the details of the study protocol were explained to each subject and their written consent was obtained. Data were collected from only four subjects, however, as the lymphocytes from two subjects did not survive in culture. Anti-Tg antibody was not detected in the serum of the four subjects who had no familial history of thyroid disease.

Media and reagents. The complete culture medium (CM) used throughout was RPMI 1640 (Sigma, St. Louis, MO) supplemented with antibiotic-antimycotic (Gibco BRL, Gaithersburg, MD) and 10% heat-inactivated FCS (Sigma). Recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF) was kindly provided by Kirin Brewery (Tokyo, Japan). rh interleukin (IL)-2, rhIL-4 and rh tumor necrosis factor (TNF)-α were purchased from PeproTech (London, UK). *Staphylococcal* enterotoxin B (SEB) was obtained from Sigma (St. Louis, MO). Human (h)Tg was obtained from Cortex Biochem, Inc. (San Leandro, CA, USA).

Generation of human monocyte-derived dendritic cells. Immature DCs (iDCs) were generated from peripheral blood as described previously (18-20). Briefly, peripheral blood mononuclear cells (PBMCs) from healthy volunteer donors were separated by Ficoll density centrifugation. The cells were then resuspended in cold PBS and allowed to adhere to 10-cm plastic dishes (Primaria, Becton Dickenson, Mountain View, CA) for 30 min at 37°C, after which the non-adherent cells were removed. Labeling the remaining adherent cells with anti-CD14 monoclonal antibody (mAb) (BD PharMingen, San Diego, CA) revealed the cell population to be >95% pure. iDCs were prepared by culturing the adherent cells in CM containing rhGM-CSF (50 ng/ml) and rhIL-4 (50 ng/ml) for 7 days; mature DCs (mDCs) were in turn prepared by incubating the iDCs with rhTNF-α (50 ng/ml) for an additional 4 days. To prepare Ag-pulsed mDCs, iDCs (10^6) were cultured with hTg (10 μg/ml) for 24 h, after which the cells were stimulated with TNF-α (50 ng/ml) for 3 days.

Flow cytometry. For surface marker analysis, cells were labeled with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mAbs against CD1a, CD83 (both from Coulter Immunology, Hialeah, FL), CD4, CD8, CD40, CD80, CD86 or HLA-DR (all from BD PharMingen). Alternatively, cells were labeled with the corresponding FITC- or PE-conjugated isotype-matched control mAb (BD PharMingen). The cells were then washed twice and suspended in cold PBS containing 25 μg/ml propidium iodide (Sigma) to exclude dead cells. Analysis of the fluorescent signal from the cells was carried out using a FACSCalibur (Becton Dickinson) with CellQuest Software (Becton Dickinson). Expression levels of cell surface products are presented as mean fluorescence intensities (MFI).

Preparation of FITC-labeled human thyroglobulin (FITC-hTg) protein. Human Tg was labeled with FITC using an antibody labeling system (FITC labeling kit, American Qualex Antibodies) according to the manufacturer's instructions. The concentration of FITC-conjugated hTg was calculated from the absorbances at 495 and 280 nm.

Endocytosis of hTg by DCs. Cells ($2\text{-}3\times10^5$ cells) were incubated for 10-60 min with various concentrations (0.1-10 μg/ml) of FITC-hTg at 37°C or 4°C. After incubation, the cells were extensively washed twice with cold PBS, and the internalization of FITC-hTg was determined by flow cytometry. Alternatively, internalization was measured following an additional chase in tracer-free CM at 37°C.

Analysis of internalization of hTg by the single cell. FITC-hTg-internalized iDCs, prepared as described above, were incubated in the absence of tracer at 37°C for 1-6 h, or were stained with PE-labeled HLA-DR. After extensive washing, the cells were cytocentrifuged onto glass slides, and the stained cells were visualized under a fluorescence microscope (Olympus IX70, Olympus, Tokyo, Japan).

Preparation of T cells. Purified T cells were prepared using a T cell negative isolation kit (Dynal, Oslo, Norway) according to the manufacturer's instructions. The purity of the resultant T cell population was typically >98% as determined by flow cytometry with anti-CD3 mAb (BD PharMingen).

Preparation of hTg-specific T cells. PBMCs obtained from healthy volunteers were cultured for 18-21 days in CM containing hTg (10 μg/ml) and rhIL-2 (100 IU/ml). The medium was changed every 7 days, and rhIL-2 was added to the culture every 3 days. Following the culture period, non-adherent cells were harvested, and the T cells were isolated as described above.

Ag presentation assay. To assess Ag-specific T cell proliferation, Ag-primed T cells (10^5) were cultured with irradiated (15 Gy from a ^{137}Cs source) autologous DCs (10^4) that had been pulsed with or without hTg (10 μg/ml) for 5 days in 96-well plates (Corning, NY). Thymidine incorporation was measured on day 5 following an 18-h pulse with [^{3}H]-thymidine (1 μCi/well, specific activity, 5 Ci/mmol; Amersham Life Science, Buckinghamshire, UK). In another experiment, the culture supernatants were collected and assayed for IFN-γ