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Combined Immunocell Therapy Using Activated Lymphocytes and Monocyte-derived Dendritic Cells for Malignant Melanoma

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Abstract. *Background: The beneficial effects of immunocell therapy, using either activated lymphocytes (ALs) or dendritic cells (DCs), in the treatment of melanoma has been demonstrated. DCs are professional antigen-presenting cells that induce cytotoxic T lymphocytes against tumor cells. DC therapy may be promising when combined with ALs. Patients and Methods: Patients with advanced melanoma, who underwent immunocell therapy with both ALs and DCs, were reviewed. DCs were pulsed with tumor lysates, peptides or both. Results: Side-effects were occasional slight fever and skin erythema. Among 8 of the 14 patients treated with immunocell therapy alone, 1 showed a mixed response (MR) and 1 prolonged stable disease (SD). In the remaining 6 patients treated with immunocell therapy and other conventional therapies, 1 CR, 1 MR and 1 prolonged SD for 24 months were observed. Conclusion: Combined immunocell therapy was well tolerated and showed a relatively high tumor response. This treatment may have therapeutic potential for some refractory malignancies.*

Immunocell therapy, in which cells of the immune system are cultured and processed *ex vivo* and administered to the patient, has been used in the treatment of human cancer, and beneficial effects have been reported for some malignancies (1). The two major fields of current interest are activated lymphocyte therapy (ALT) and dendritic cell vaccination therapy (DCT). ALT was first introduced by Rosenberg *et al*. in the late 1980s (2). However, treatment was accompanied by severe toxicity due to the coadministration of high dosages of interleukin-2 (IL-2) (1). A number of researchers, nevertheless, persevered with variations of ALT, and trials have now led to its use by the repeated administration of T cells, activated and proliferated by culture of peripheral blood mononuclear cells under stimulation with immobilized CD3-antibody and IL-2 (3-5). The efficacy of this therapy and its fewer side-effects in some malignancies have been reported, and its use for cancer has now spread in Japan, in some medical institutions under approval of the Ministry of Health, Labor and Welfare as "highly advanced medical technology".

Recent attention has also been focused on DCT. The efficacy of this treatment in melanoma patients was first reported by Nestle *et al*., and subsequently confirmed by several other groups (6-8). In this system, dendritic cells (DCs) are induced to differentiate from peripheral blood monocytes using granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), then pulsed with tumor lysates or peptides and used as a DC vaccine.

Despite much effort, the efficacy of chemotherapy for melanoma remains markedly limited and patients with metastatic melanoma have a very poor prognosis (9). Alternative therapeutic approaches have been sought. Melanoma is considered to have immunogenic properties and has, in fact, been the lead subject in the study of immunocell therapy, although limited, beneficial effects in melanoma patients have been demonstrated with both ALT and DCT. DCs are professional antigen-presenting cells that induce cytotoxic T lymphocytes against tumor cells. DCT may be promising when combined with ALT, in which a large number of activated T lymphocytes is transferred.

Several antigens on melanoma, recognized by cytotoxic T lymphocytes, have been identified, beginning with Boon *et al*'s report of an antigenic peptide on HLA-A1 melanoma,

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Table I. *Antigens used to pulse DCs.*

ID	DCs pulsed with	Antigen	HLA specificity	Epitope	Antigen	HLA specificity	Epitope
1	Lysate and Peptide	MAGE-1	A-24	NYKHCFPEI	MAGE-3	A-24	IMPKAGLLI
2	Peptide	MAGE-1	A-24	NYKHCFPEI	MAGE-3	A-24	IMPKAGLLI
3	Lysate	-	-	-	-	-	-
4	Peptide	MAGE-1	A-24	NYKHCFPEI	MAGE-3	A-24	IMPKAGLLI
5	Peptide	NYESO-1	A-31	LAAQERRVPR	TRP-2-2	A-31	LLGPGRPYR
6	Peptide	MAGE-3	A-24	IMPKAGLLI	gp100	A-24	VYFFLPDHL
7	Peptide	MART-1mod.	A-2	ELAGIGILTV	gp100	A-24	VYFFLPDHL
8	Peptide	MAGE-3	A-24	IMPKAGLLI	gp100	A-24	VYFFLPDHL
9	Lysate	-	-	-	-	-	-
10	Peptide	MART-1mod.	A-2	ELAGIGILTV	gp100	A-2	KTWGQYWQV
11	Peptide	MART-1mod.	A-2	ELAGIGILTV	TRP-2-2	A-31	LLGPGRPYR
12	Peptide	TRP-2-2	A-31	LLGPGRPYR	NYESO-1	A-31	LAAQERRVPR
13	Lysate and Peptide	MAGE-3	A-2	FLWGPRALV	gp100	A-2	KTWGQYWQV
14	Lysate and Peptide	MAGE-3	A-2	FLWGPRALV	Mart-1	A-2	AAGIGILTV

termed MAGE1 (10), and subsequently melanocyte-differentiation antigens such as MART-1 and gp100 (11).

Against this background, we have established four private clinics specializing in immunocell therapy (ALT and DCT) over the last 6 years. The clinics are equipped with a clean room enabling aseptic handling of living cells, and can produce sufficient cells for administration to large numbers of patients (1, 12, 13).

In this retrospective study, we investigated outcomes in 14 melanoma patients who received ALT as well as DCT. The beneficial effects of this combined immunocell therapy are discussed.

Materials and Methods

Patients and treatment. Patients with histologically confirmed melanoma, who underwent immunocell therapy with autologous activated lymphocytes (ALs) and DCs at the four clinics of the Seta Group between April 1999 and November 2004, were reviewed. Twenty consecutive patients were treated by immunocell therapy, among whom 2 patients elected to restrict treatment to only 1 or 2 infusions of ALs and DCs. The remaining 18 patients underwent at least 1 course of treatment consisting of 6 infusions. Of these, 14 had evaluable tumors and were used for the investigation of outcome. Written informed consent was obtained from all the patients before the start of therapy. ALs ($3\text{-}10\times10^9$) and DCs ($1\text{-}10\times10^6$) were infused intravenously or intratumorally at an interval of about 2 weeks. The patients also received conventional therapies in combination where indicated. The cell processing and immunocell therapy procedures used were approved by the Ethical Committee of our institution.

Generation of ALs and DCs. Mononuclear cells (MNCs) were collected from 45 ml of peripheral blood using a Vacutainer (Becton Dickinson; NJ, USA) in 11 of the 14 patients, and by leukapheresis in the remaining 3 (cases 3, 4 and 5). MNCs were allowed to adhere to the plastic culture flask. Non-adherent cells were collected and cultured for 2 weeks with 700 IU/ml recombinant IL-2 (Proleukin^s, Chiron; Amsterdam, Netherlands), then activation with immobilized anti-CD3 monoclonal antibody (Janssen-Kyowa; Tokyo, Japan) using HyMedium 930 (Kohjin Bio; Saitama, Japan) containing 1% autologous serum. After culture for 14 days, $3\text{-}10\times10^9$ cells were harvested and suspended in 100 ml of saline. To generate DCs, adherent cells were cultured in the presence of 50 ng/ml GM-CSF (Primmune Corp; Osaka, Japan) and 50 ng/ml IL-4 (Primmune Corp) for 6 days to generate DCs. Twenty-four hours prior to administration, the DCs were cultured with appropriate antigens of either 100 μg/ml of tumor lysate or 10 μg/ml of synthetic peptide, and approximately $1\text{-}10\times10^6$ DCs were harvested for injection.

Preparation of tumor lysates and peptides. Tumor lysates and peptides were used as the tumor antigen. To prepare lysates, the tumor mass from a surgically resected specimen was cleaned of non-malignant tissues with a scapel, and lysed by passage through 3 freeze/thaw cycles in liquid nitrogen and a 37˚C water bath. The lysate was centrifuged and the supernatants were passed through a 0.22-μm filter (Millipore Corporation; MA, USA). The protein content of the lysates was determined and aliquots were stored at –80˚C until use. Peptides used for pulsing DCs were purchased from Qiagen K.K. (Tokyo, Japan). The type of peptides used, their epitope and HLA specificities are shown in Table I (11).

Evaluation of tumor response. Tumor lesions were evaluated by physical or radiographic examination. A complete response (CR) was defined as the disappearance of all lesions; a partial response (PR) as a reduction of more than 50% in the product of the bidimensional diameters; progressive disease (PD) as the appearance of new lesions or an increase of more than 25% in the product of the bidimensional diameter of an existing lesion; and stable disease (SD) as neither PR nor PD. Prolonged SD was defined as SD which remained unchanged for more than 6 months.

Table II. *Patient characteristics.*

ID	Age	Sex	Stage	Primary site	Metastases	Previous therapies	PS**
1	50	F	4	paranasal sinus	bone, skin spleen	S,R,C*	2
2	44	F	4	nasal cavity	skin, liver lung	R	2
3	37	M	4	skin	skin, brain, lymph node	S	0
4	42	F	4	vagina	lymph node	S, C	0
5	61	M	4	unknown	lung	none	0
6	71	M	4	esophagus	skin	S	2
7	55	F	3	vagina	none	R, C	0
8	68	F	4	paranasal sinus	adrenal gland, lymph node	none	2
9	36	M	4	neck	brain, spinal cord	S,R,C	1
10	60	F	4	skin	lung, lymph node	S, C	0
11	36	M	4	skin	bone, skin spleen	S, C	1
12	49	F	4	nasal cavity	skin, lymph node	C	1
13	53	F	4	head	skin, bladder	S, C	0
14	32	F	4	skin	skin, lung, liver	C	0

*S: sugery, R: radiotherapy, C: chemotherapy
**performance status

Table III. *Tumor response and outcome.*

ID	Combined therapy	DCs pulsed with	No. of infusions	Clinical response (months)	Outcome (days)
1	none	Lysate and Peptide	20	SD	Dead (404)
2	none	Peptide	31	MR (23mo.)	Dead (1039)
3	none	Lysate	6	PD	Alive (220)
4	none	Peptide	6	PD	Alive (99)
5	none	Peptide	12	prolonged SD (9 mo.)	Alive (302)
6	none	Peptide	6	PD	Dead (147)
7	none	Peptide	10	PD	Dead (116)
8	none	Peptide	7	PD	Dead (101)
9	IFN-β	Lysate	6	SD	Alive (93)
10	C, IFN-β	Peptide	6	CR (14 mo.)	Alive (452)
11	IFN-β	Peptide	8	PD	Dead (271)
12	IFN-β	Peptide	29	MR→PR (14 mo.)	Alive (441)
13	C, IFN-β	Lysate and Peptide	8	PD	Dead (245)
14	C	Lysate and Peptide	21	Prolonged SD (24 mo.)	Alive (741)

Cases in which PR was observed in some tumors while new lesions appeared simultaneously were defined as a mixed response (MR).

Results

Patients' characteristics. The patient characteristics are shown in Table II. There were 9 female and 5 male patients with ages in the range 36-72 years. The disease stage was 4 in 13 patients with distant metastases at various sites, and 3 in 1 patient who had locally advanced melanoma in the vagina with lymph node metastases. Twelve of the 14 patients had previously undergone surgery, radiotherapy or chemotherapy. The performance status was 0 in 7, 1 in 3 and 2 in 4 patients.

Clinical response. Eight of the 14 patients were treated with immunocell therapy alone (patients 1-8) while the others were treated with immunocell and other therapies (patients 9-14) (Table III), namely injection of interferon β (IFN-β) in 3, conventional chemotherapy in 1, and injection of interferon β and conventional chemotherapy in 2. DCs were pulsed with peptides alone for 9 patients, tumor lysate alone for 2 and both peptides and lysate for 3. The patients received 6 or more infusions of ALs and DCs.

The tumor response to therapy was determined after 1 course of immunocell therapy, which consisted of 6 infusions of ALs and DCs. Among the 8 patients treated with immunotherapy alone, 1 MR and 1 prolonged SD were observed. In patient 2, a liver metastasis disappeared following immunocell therapy alone (Figure 1), although new lesions appeared in the skin during the first course of therapy. No further progression was seen for 23 months, at which time the metastatic tumor in the liver reappeared and progressed. This patient did not undergo chemotherapy, and remained alive for 1,039 days after the first immunocell therapy. The patient with prolonged SD has remained stable for 9 months up to the time of writing (patient 5). In 6 patients treated with immunocell therapy as well as IFN-β or chemotherapy, 1 CR, 1 MR and 1 prolonged SD for 24 months were observed. Patient 10 received immunocell therapy in combination with conventional chemotherapy (dacarbazine, nimustine hydrochloride, vincristine sulfate and IFN-β), and showed the complete disappearance of metastatic tumors in the inguinal lymph nodes after 6 infusions of ALs and DCs. This patient with CR is still free of tumors at 14 months after therapy without additional immunocell therapy or chemotherapy. In patient 12, multiple metastatic sites in the skin regressed or disappeared following cotreatment with immunocell therapy and IFN-β, while new lesions appeared simultaneously (Figure 2). In this patient, all sites underwent regression or disappeared on continuation of cotreatment without the appearance of new lesions.

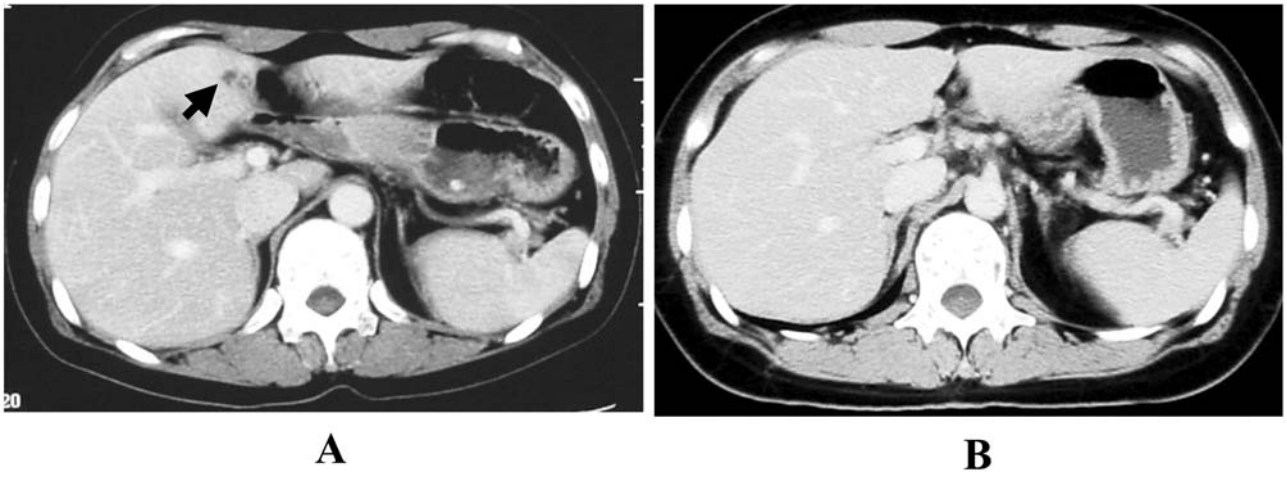

Figure 1. *Disappearance of a liver metastasis in a patient with immunocell therapy alone. CT scan in Patient 2 on 19 April, 2000, showed a small metastatic lesion in the liver (A). The lesion could not be seen on rescan on 25 May, 2000 (B).*

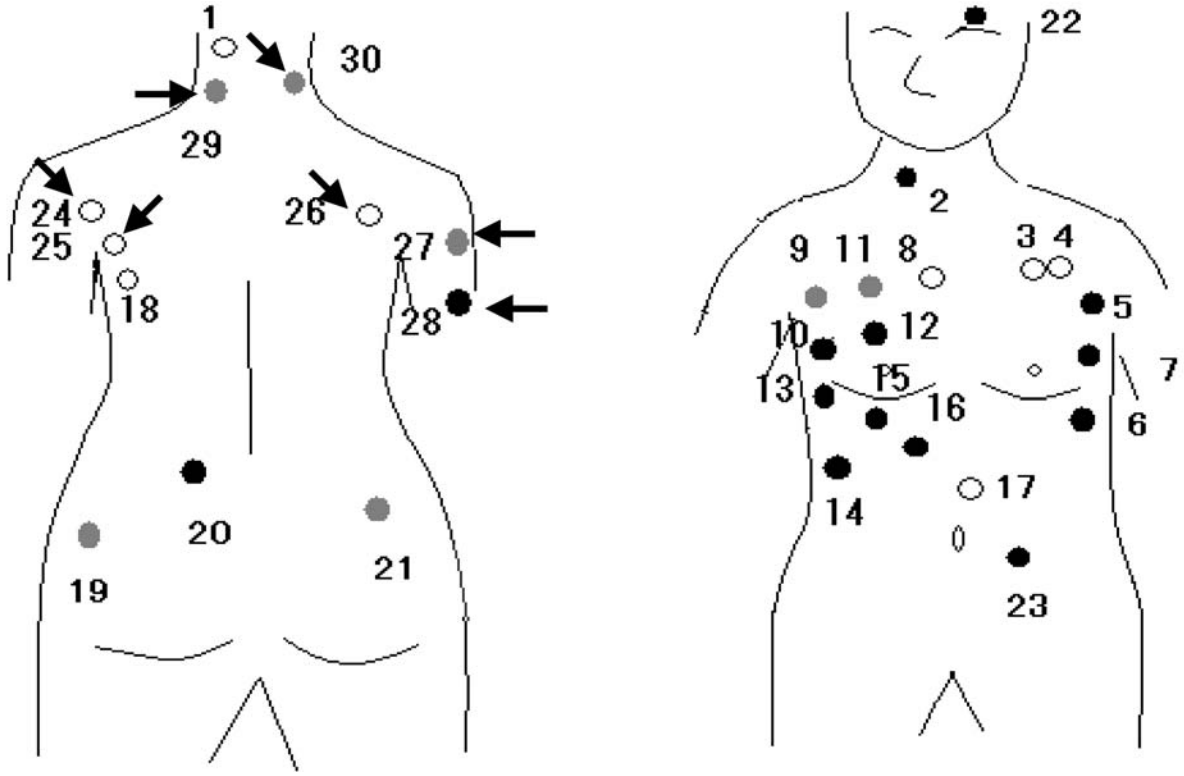

Figure 2. *Regression or disappearance of multiple metastatic sites in the skin and simultaneous appearance of new sites in a patient on cotreatment with immunocell therapy and IFN-β. In patient 12, tumors completely regressed (●), remarkably regressed (○) or partially regressed (●) with 1 course of treatment. Tumors 24-30 (→) were lesions which newly appeared during treatment.*

The only side-effects observed were occasional slight fever for 1 day after immunocell therapy. In patient 7 with CR, skin erythema appeared over a large area of the body after every immunocell infusion. No other side-effects were observed in any patient. The survival time was no less than 99 days from the start of treatment in any patient. Maximum survival at days was achieved by patient 2 with MR.

Discussion

Although previous clinical trials of treatment for melanoma with either DCT or ALT alone have shown immunological and clinical effects, efficacy has been somewhat limited (6-8). DCs are known to be potent antigen-presenting cells in the induction of tumor-specific CTL in animals as well as humans. The generation of a potent CTL response requires the presence of helper T lymphocytes (14, 15). Owing to the diminished immune cell function in patients with cancer, including that of DCs and T lymphocytes, and the resulting immunological tolerance of tumors that this deficiency leads to (16), it is probable that the tumor antigen-pulsed DCs administered to these patients may not have had sufficient function *in vivo*. Our previous study showed that the type 1 immune response is significantly suppressed in cancer patients (17). Some trials used an injection of keyhole limpet hemocyanin or IL-2 as adjuvant for DCT (6, 8), but these were accompanied by moderate adverse effects. Flow cytomeric analyses showed that more than 90% of ALs used in our treatment consisted of CD8+ or CD4+ T lymphocytes, which produce cytokines of the type 1 immune response such as IL-2 and interferon γ. ALT may thus help to enhance the type 1 immune response. In addition, ALT itself has been demonstrated to have beneficial effects against melanoma and other malignancies (2). We, therefore, hypothesized that DCT combined with ALT may be promising for the treatment of melanoma.

In this study, no severe side-effects were observed and favorable effects were seen in some patients. In 8 patients with immunocell therapy alone, 1 MR was observed. Although previous studies with DCT alone have also reported PR or MR in a few patients with melanoma, the present results indicate the superiority of cotreatment with ALT and DCT over DCT alone, including the putative demonstration of MR of 23 months' duration and patient survival as long as 1039 days. In the group with the combination of immunocell and other therapies, 1 CR and 1 MR were observed, both notably of more than 14 months' duration until the time of writing. Moreover, the SD observed in 1 patient remained for 21 months. Tumor response to this therapy, if it occurs, appears to be maintained for an extended period. Chemotherapy for melanoma is reported to have lower efficacy, with response rates of about 30% (9). Against this, the present results suggest that the combination of immunocell therapies results in a higher tumor response rate and longer duration of response. The median survival time in one study with a large number of patients with metastatic melanoma was reported to be 7.5 months (18). In the present study, in contrast, survival from the start of immunocell therapy was more than 8 months in 8 of the 14 patients, while 2 patients have shown survival of more than 24 months. Moreover, 9 of the 14 patients had undergone chemotherapy before our therapy started. The survival time of patients in this study is, therefore, considered to be relatively good.

In the present study, MR was observed in 2 patients. In patient 12, multiple existing metastatic sites in the skin regressed or disappeared with treatment, although new skin lesions appeared simultaneously. MR was not frequently observed in chemotherapy. MR has also been reported in previous studies of DCT for melanoma (8). Specific mechanisms of MR, if such in fact exist, might include the generation of a CTL immune response in tumors with established vascularity but not in micrometastatic tumors; alternatively, cells in regressing tumors might strongly express major histocompatibility complex (MHC), the main molecule on tumor cells recognized by CTL, whereas cells in newly arising tumors may show less expression. Research into the mechanisms of MR is ongoing in our laboratory.

In conclusion, the present study suggests that combined immunocell therapy has few toxic effects and a greater tumor response than single immunocell therapy alone. These findings indicate that combined immunocell therapy may have therapeutic potential in the treatment of some refractory malignancies.

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