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Vaccination with IL-12 gene-modified autologous melanoma cells: preclinical results and a first clinical phase I study

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Cytokine gene transfer into tumor cells has been shown to mediate tumor regression and antimetastatic effects in several animal models via immunomodulation. Therefore, clinical protocols have been developed to treat cancer patients with cytokine gene-modified tumor cells. We inserted the genes coding for the p35 and p40 chain of interleukin-12 (IL-12) in two independent eukaryotic expression vectors and transduced melanoma cells of 15 different primary tumor cultures with both plasmids by a ballistic gene transfer approach. Secreted IL-12 demonstrated strong bioactivity by inducing interferon-γ release from peripheral blood lymphocytes upon coculture with cell culture supernatants after IL-12 gene transfer which could at least partly be blocked by IL-12-specific antisera. Further enrichment of transduced tumor cells by magnetic separation directly after gene transfer increased cytokine secretion from a mean of 119 pg in the unsorted to 507 pg IL-12 (24 h/10^6 cells) in the magnetically enriched cell fraction. Irradiation of these cells led to a further elevation of secreted IL-12 (mean 987 pg). Elevated IL-12 levels were detected over 7 days after irradiation in vitro. In a subsequent first clinical phase I study six patients with metastatic melanoma were vaccinated with autologous, interleukin-12 gene-modified tumor cells. Melanoma cells were expanded in vitro from surgically removed metastases, transduced by ballistic gene transfer, irradiated and were then injected subcutaneously (s.c.) at weekly intervals. Clinically, there was no major toxicity except for mild fever. All patients completed more than four s.c. vaccinations over 6 weeks and were eligible for immunological evaluation. Post-vaccination, peripheral mononuclear cells were found to contain an increased number of tumor-reactive proliferative as well as cytolytic cells as determined by a limiting dilution analysis in two patients. Two patients developed DTH reactivity against autologous melanoma cells and one had a minor clinical response. Biopsies taken from that patient's metastases revealed a heavy infiltration of CD4$^+$ and CD8$^+$ T lymphocytes. In conclusion, vaccination induced immunological changes even in a group of advanced, terminally ill patients. These changes can be interpreted as an increased antitumor immune response.

Keywords: *cytokine gene transfer; DTH reactivity; IL-12 secretion*

Introduction

Of all the therapies developed to treat solid tumors, those for malignant melanoma are among the least likely to be successful. Treatment schedules including radiation, chemo- and immunotherapy as well as combinations have no significant impact on the overall survival of patients.[1,2] On the other hand, malignant melanoma is considered to be a prototype of an immunogenic tumor. Clinical observations in patients with melanoma reveal that spontaneous, partial or complete tumor regressions, although rare, do occur and that the disease is often accompanied by concomitant destruction of melanocytes in benign lesions, leading to clinical phenomena such as halo nevi, uveitis and vitiligo. It is believed that the immune attack of infiltrating lymphocytes against melanoma cells may be responsible for these observed phenomena.[3]

Recently, cytolytic T lymphocytes (CTL) which recognize and destroy tumor cells have been isolated from blood or from tumor infiltrating lymphocytes of melanoma patients by numerous investigators. Furthermore, it has been demonstrated that such HLA-restricted T lymphocytes are capable of mediating impressive tumor regression *in vivo*.[3,4] However, the induction of tumor-specific immunity *in vivo* has been considered difficult to achieve.

Local secretion of cytokines including IL-2, IL-4, TNF and others has been achieved in murine tumor models by introducing genes directly into tumor cells. In contrast to systemic application of these cytokines, cytokine gene transfer led to suppressed tumor growth and induction of antitumor immunity in certain models.[5,6] It is believed that local secretion of cytokines might activate T helper function at the tumor site which is essential for the induction of *in vivo* antitumor immunity. Introduction of the

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IL-12 gene into tumor cells or into fibroblasts subsequently mixed with autologous tumor cells has been shown to elicit potent growth inhibitory as well as antimetastatic effects. Furthermore, IL-12 gene transfer induced a T_{H1} response and generated a strong antitumor immunity.[7–16]

IL-12 is a heterodimeric cytokine originally cloned from B lymphoblastoid cell lines. The cDNAs encoding the two IL-12 subunits are unrelated and encode for two distinct proteins which have molecular masses of 35 kDa and 40 kDa. Monocytic cells appear to be the major source of IL-12, but other cells such as mast cells, B cells, keratinocytes and dendritic cells can produce IL-12. Receptors for IL-12 were described on NK and T cells which respond with enhanced proliferation and increased cytolytic activity. Furthermore, cytokines such as IFN-γ are released by T cells upon encountering IL-12.[17–19]

Based on successful animal studies using gene-modified tumor cells, various clinical protocols for the treatment of human cancer have been developed in recent years. A vaccination therapy using gene-modified autologous tumor cells is especially suitable for the treatment of melanoma and is thought to be a means of presenting potentially all individual tumor antigens to the host immune system.[20,21] To investigate the autologous vaccination approach, we manipulated autologous melanoma cells to overexpress IL-12 using a mammalian expression plasmid vector system and a ballistomagnetic gene transfer technique. A first clinical phase I trial was carried out to evaluate the feasibility and the clinical toxicity of such an approach. Furthermore, we report here on achieving modulation of the immune response in a subset of patients with metastatic melanoma in an advanced stage after immunization with the described IL-12 gene-modified, autologous tumor cell vaccine.

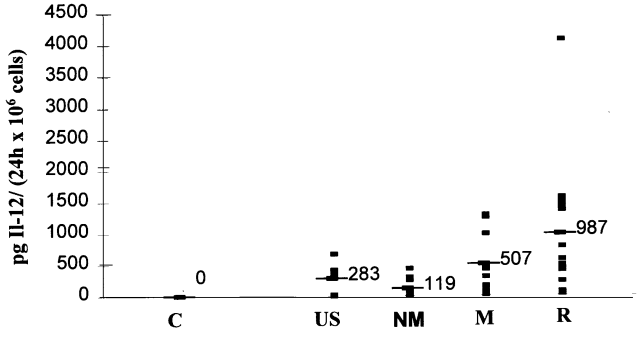

Figure 1 *IL-12 secretion after magneto-ballistic transfer. Release of pg IL-12 (24 h × 10^6 cells) into cell culture supernatant in nontransfected melanoma cell cultures before gene transfer (C) (mean 0 pg IL-12), in cell supernatants after ballistic gene transfer without magnetic separation (US) (mean 283 pg IL-12), after separation in the nonmagnetic cell fraction (NM) (mean 119 pg IL-12) and the magnetic fraction (M) (mean 507 pg IL-12) and after irradiation (100 Gy) of the magnetic cell fraction (R) (mean 987 pg IL-12) as determined in 15 melanoma cell cultures each by ELISA.*

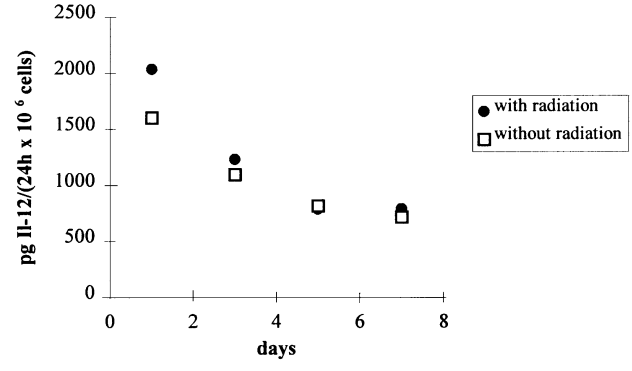

Figure 2 *IL-12 secretion over 7 days. IL-12 secretion (mean) after ballistic gene transfer of four human melanoma cell cultures of irradiated (100 Gy) and nonirradiated cells. The experiments were performed in duplicates.*

Results

Preclinical assessment of IL-12 secretion and biological activity

Primary melanoma cell cultures from 15 metastases of patients were analyzed for the release of IL-12 from tumor cells after gene transfer. Gold particles coated with p35 and p40 encoding vector constructs were used for ballistic transfer. Cell fractions were magnetically separated and aliquots of all fractions were analyzed for IL-12 secretion after 24 h by ELISA. Nontransfected cells (C) did not secrete any IL-12 (Figure 1). The whole cell mixture after ballistic transfer without separation (US) released a mean of 283 pg IL-12 (24 h; 10^6 cells) which increased to 507 pg IL-12 (24 h; 10^6 cells) after magnetic separation (M) compared with 119 pg IL-12 (24 h; 10^6 cells) in the nonmagnetic cell fraction (NM). Irradiation (100 Gy) of the magnetic fraction (R) further increased IL-12 secretion to 987 pg IL-12 (24 h; 10^6 cells) (Figure 1). More detailed analysis of IL-12 secretion by ELISA in four selected melanoma cell cultures after gene transfer demonstrated significant secretion of IL-12 over more than 7 days with no difference between irradiated and nonirradiated cells (Figure 2). No homodimers were detected by ELISA. In order to test the bioactivity of the IL-12 secreted, interferon-γ release from PBL obtained from two healthy donors was analyzed. Both PBL preparations secreted IFN-γ in a dose-dependent fashion after the addition of recombinant IL-12 (Figure 3) which could be blocked by a goat anti-human IL-12 antiserum (2 μg/ml). An even higher release of IFN-γ was observed upon addition of cell culture supernatant of IL-12-transfected melanoma cells. That effect could only partly be blocked (Figure 3). However, supernatants of nontransfected cells had no effect on IFN-γ secretion (not shown). Proliferation kinetics of five human melanoma cell lines were not affected when recombinant IL-12 (1 pg/ml to 1 μg/ml) was added for up to 7 days (not shown).

Clinical assessment of the course of disease, the immune status, adverse effects and toxicity

Six patients were enrolled in the clinical phase I study (Table 1). All patients suffered from advanced metastatic melanoma, with the mean disease-free interval from excision of the primary tumor to the first distant metastasis amounting to 21.5 months (range 2–73 months; not shown). Since all patients had previously received various conventional oncological treatment regimens, an additional 8.3 months had elapsed on average before vaccination could be started (not shown). Vaccination treat-

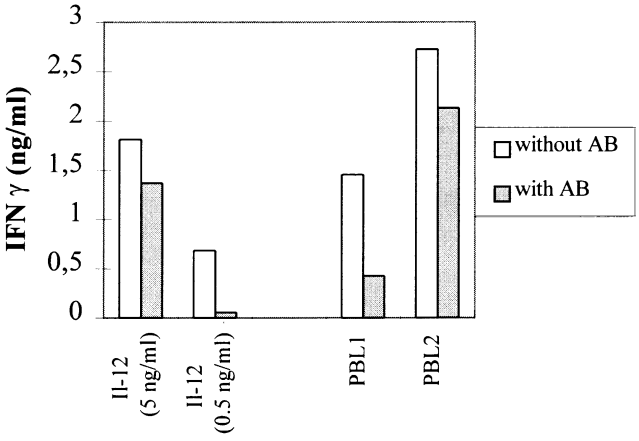

Figure 3 *IL-12 induced interferon-γ secretion. PBLs from two different healthy donors (PBL1 and PBL2) were incubated with the supernatants of IL-12-transfected melanoma cells (right) or recombinant human IL-12 (left). Specific goat anti-human IL-12 antiserum (AB) was used to suppress IL-12-mediated IFN-γ production. Determinations were done in duplicate and repeated twice.*

ment could be begun between 2 weeks and 3 months after surgical removal of the metastases.

Characterization of vaccines: In order to evaluate possible interactions between immune effector cells and melanoma cells used as tumor vaccines, we characterized the autologous melanoma cells in regard to the expression of cell surface markers (HLA-A,-B,-C, HLA-A2, HLA-DR, HLA-DQ and ICAM-1) by FACS, the release of immunosuppressive mediators (IL-10 and TGF-β1) by ELISA and tumor antigens known to be recognized by T cells (tyrosinase; gp100, MART-1, TRP-1, TRP-2, MAGE-1 and MAGE-3) by RT-PCR. All melanoma cells used for vaccination demonstrated high reactivity with MAb recognizing HLA class I and class II (Table 2) as they did with ICAM-1 (not shown). PBL from patients SK, RE and HA were typed HLA-A2 positive (Tables 1 and 2). HLA-DR expression on melanoma cells was detected in two patients (Nos 1 and 5; Table 2). TGF-β1 secretion by tumor cells varied between 0.37 and 1.42 ng/ml/10^5 cells/24 h (Table 2) whereas IL-10 secretion was not detectable in any of the cell lines (not shown). Expression of tumor antigens was determined by RT-PCR and demonstrated the presence of tyrosinase and gp100 in four of five melanoma lines analyzed. An abundant amount of MART-1/Melan-A was detected in three patients' tumor cells (RE, GG and HA). Melanosomal proteins TRP-1 and TRP-2 were detected on the mRNA level in three patients each, whereas MAGE-1 and MAGE-3 were not detectable by RT-PCR in any of the six lines.

Vaccination and clinical effects: Patients received between 1×10^5 and 9.6×10^6 autologous, IL-12 gene-modified melanoma cells per vaccination with the total number of gene-modified cells administered ranging between 4.6×10^6 and 1.6×10^7 (Table 3). Vaccinations were tolerated well by all patients without any signs of toxicity. No erythema, swelling or induration at the injection site was detectable in any of the patients. However, two patients showed superficial erythematic reactions of cutaneous tumors far from the vaccination sites, 2 days after the fourth vaccination (SK and GG). DTH reactiviy was negative in all patients before vaccination and remained so, except SK and GG. Mild fever (grade I–II) and mild flu-like symptoms were observed in two patients (HA and SK) with temperatures up to 39°C, lasting up to 24 h after immunization. All six patients received the first four immunizations and were evaluable in week 5. Two patients (SK and MK) received five, one (HA) six and another (GG) eight immunizations before disease progression became obvious (Table 3). No major clinical response (CR, PR) was observed in any patient. Three patients (HA, MK and GG) showed a stabilization of the disease (4 months, 4 months, and 3 months) and one a minor response (SK) with the regression of some cutaneous metastases over 3 months. Two patients (RE and OF) received a fourth vaccination, but further immunization was terminated at the next hospital visit in both cases because of tumor progression. Patients died an average of 5.5+ months after initiation of vaccination with IL-12 gene-modified tumor cells; two patients (GG and HA) were still alive 10 and 12 months, respectively, after the beginning of immunization.

Immunological evaluation

Delayed-type hypersensitivity (DTH): T cell-mediated skin reactivity towards common recall antigens such as bac-

Table 1 Patient characteristics and DTH reactivity (Multitest Merieux skin test (sum of total diameter given in mm/No. of reactive antigens)) before and after vaccination

Patient					*DTH reactivity (Multitest Merieux)*	
No.	*Initials*	*Age/Sex*	*HLA typing*	*Karnofsky index*	*Before gene therapy*	*After fifth vaccination*
1	MK	37/M	A1, A11, B8, B35, Cw4, CW7, DR1, DR17	>70	14.0 mm/4	11.0 mm/3
2	SK	28/M	A2, B38(16), B60 (40), Cw3 DR1, DR15, DQ5, DQ6	>70	14.0 mm/3	11.0 mm/3
3	OF	58/M	A3, A29(19), B27, B44(12), Cw2, DR7, DR11(5)	<70	0 mm/0	0 mm/0
4	RE	49/F	A2, A11, B22, B75, Cw1, Cw3, DR1, DR15	>70	4.0 mm/1	8.0 mm/1
5	GG	47/M	A1, A3, B57(17), B62(15), Cw3, Cw6, DR3, DR4	>70	24.0 mm/3	20.0 mm/3
6	HA	46/M	A2, A31(19), B51(5), B57(17), Cw6, Cw7	>70	2.0 mm/1	26.0 mm/4

Table 2 Characterization of melanoma cell vaccines used for the immunization

Patients		*Surface markers (MFI)*				*TGF-β1 release (ng/ml/10⁵ cells/24 h)*	*Expression of tumor antigens (RT-PCR)*						
No.	*Initials*	*HLA-A,B,C*	*HLA-A2*	*HLA-DR*	*HLA-DQ*		*Tyrosinase*	*gp100*	*MART/1*	*TRP-1*	*TRP-2*	*MAGE-1*	*MAGE-3*
1	MK	71.4	ND	60.1	2.3	0.53	++	++	++	+/−	−−	−−	−−
2	SK	118.2	13.5	1.3	1.0	1.42	+/−	−−	−−	−−	−−	−−	−−
3	OF	51.1	1.1	1.0	1.1	0.66	+	++	−−	+/−	+	−−	−−
4	RE	67.9	9.4	1.0	1.0	0.37	++	++	+	+	++	−−	−−
5	GG	89.5	1.1	14.3	1.2	0.75	++	++	++	−−	−−	−−	−−
6	HA	33.1	11.3	1.1	1.3	0.54	++	+++	+	+	++	−−	−−

MFI (mean fluorescence index) = fluorescence of specific stained cells / fluorescence of negative control staining; ND, not done; −− not detectable; +, specific amplification; +/−, questionable, at the detection level.

Table 3 Number of gene-modified cells used for each vaccination administered into multiple injection sites. Total number of gene-modified melanoma cells given were calculated after last vaccination

Patient		*No. cells used for vaccination*								
No.	*Initials*	*First dose*	*Second dose*	*Third dose*	*Fourth dose*	*Fifth dose*	*Sixth dose*	*Seventh dose*	*Eighth dose*	*Total no. of cells administered*
1	MK	8×10^5	1×10^5	1.2×10^5	2.6×10^6	3.2×10^6	Not given	Not given	Not given	6.8×10^6
2	SK	1×10^6	7×10^5	4×10^5	5×10^5	1.4×10^5	Not given	Not given	Not given	2.7×10^6
3	OF	1.1×10^6	1.2×10^6	5×10^5	4×10^6	Not given	Not given	Not given	Not given	6.8×10^6
4	ER	3×10^6	2×10^6	1.1×10^6	9.6×10^6	Not given	Not given	Not given	Not given	1.6×10^7
5	GG	5×10^6	3×10^6	4×10^5	1×10^6	2.7×10^6	6.3×10^6	1.7×10^6	1.2×10^6	1.4×10^7
6	HA	6×10^5	1×10^5	5×10^5	6×10^5	1.7×10^6	1.1×10^6	Not given	Not given	4.6×10^6

terial antigens, tetanus toxoid etc, as determined by the Multitest Merieux and reflecting the overall immunological status of the patients, was already dramatically reduced in patient OF before therapy (Table 1). DTH reactivity towards autologous melanoma cell lysates was negative in all patients before vaccination and remained nonreactive in four patients over the course of vaccination. Specific DTH reactivity after intradermal injection of autologous melanoma cell *versus* peripheral blood cell lysates (control) was observed in two patients (HA and GG) before the fifth immunization. No DTH reactivity was noted at the start of the therapy.

Frequency analysis of tumor-reactive lymphocytes: The precursor frequencies of tumor-reactive lymphocytes in PBMC before and after the fourth vaccinations were analyzed by setting up limiting dilution (LD) microcultures in order to obtain mixed lymphocyte tumor cultures (MLTC) in a statistically sufficient number and distribution for a mathematical evaluation. As a side-product, we generated several T cell lines at the end of the LD culture periods. Quantitative results of lytic clones from PBMC obtained before the first and after the fourth vaccination could be determined in two patients after coculture with autologous melanoma cells over 25 days *in vitro* (Table 4). In one patient (No. 6) not enough cytolytic clones were generated to allow mathematical calculation; in patient Nos 1, 2 and 3 no specific antimelanoma cytolytic activity was detectable before and after vaccination in peripheral blood. However, unspecific NK-like activity was dramatically increased in all patients (not shown). Two patients (Nos 4 and 5) had an increase of tumor-reactive proliferation and specific antimelanoma cytotoxicity in responder lymphocytes with a two-fold increase in RE (No. 4) and a more than 7.5-fold increase in GG (No. 5) (Table 4). Further culture of the T cell lines demonstrated that cytotoxicity could be blocked by anti-HLA class I antibodies (W6/32). However, no known melanoma-associated peptide presented by HLA-A2 for RE or presented by HLA-A1 or HLA-A3 for patient GG was recognized by the T cell lines as determined by ELISPOT analysis (not shown).

Immunohistochemical analysis of tumor specimens taken over the clinical course: Metastases taken before vaccination and after the third to fifth vaccinations were available in four patients for histological characterization of the infiltrate (Table 5A) and the expression of melanoma-associated antigens (Table 5B). Neither MAGE-1 and MAGE-3 proteins nor CD56-positive cells were identified in any tumor tissue. The antigen expression pattern was not altered in three of four patients. Expression of tyrosinase and Melan-A was highly reduced in the metastasis taken after immunization in patient MK (Table 5B). CD1a-positive cells were found in sparse amounts in two of four patients with no change upon immunization. CD4 and CD8 expressing T cells were already present in small numbers around and infiltrating the metastases before

Table 4 LD cultures with lytic activity against autologous melanoma cells[a] and frequency analysis of antimelanoma lytic cells in PBMC

Patients	*Before vaccination*	*Frequency in PBMC*	*After vaccination*	*Frequency in PBMC*
4	3 (2)	1/201,117 (1/234,676)	8 (7)	1/100,396 (1/115,550)
6	5 (3)	[b]	4 (2)	[b]
5	5 (0)	1/135,065	61 (20)	1/9,440 (1/18,461)

[a]Number of LD cultures with lytic activity either against autologous melanoma only or against autologous melanoma and K562 in one LD culture.
[b]Did not follow single-hit kinetics, with *P* values >0.1.
In parenthesis, LD cultures and frequency, respectively, with specific lytic activity against autologous melanoma cells only.

Table 5 Immunohistological characterization of the immunological effector cells (A) and the expression of melanoma-associated antigens recognized by T cells (B) in metastases taken before and after vaccination with IL-12 modified cells

	MK		*SK*		*RE*		*GG*	
	Before	*3. Vacc*	*Before*	*4. Vacc*	*Before*	*4. Vacc*	*Before*	*5. Vacc*
A. Immunological effectors								
CD4	(+)	++	+	+	−−	++	+/++	+/++
CD8	+	+++/−	+	++	−−	++	++	+++
CD25	(+)	+	+	++	−−	(+)	+	(+)
CD56	−−	−−	−−	−−	−−	−−	−−	−−
CD1a	(+)	+	−−	−−	−−	−−	(+)	(+)
CD68	(+)	++	+	++	−−	−−	+/++	++
B. Melanoma antigens								
Tyrosinase	100%	<5%	<5%	0%	0%	0%	100%	100%
TRP-1	15%	0%	0%	0%	100%	100%	0%	0%
Melan-A	100%	<5%	<5%	0%	100%	100%	100%	100%
gp100	15%	20%	<5%	0%	100%	100%	80%	70%
MAGE-1	0%	0%	0%	0%	0%	0%	0%	0%
MAGE-3	0%	0%	0%	0%	0%	0%	0%	0%

(+), single infiltrating cells detectable; +, sparse infiltration; ++, clusters of infiltrating cells; +++, heavy infiltration of the tumor; –, not detectable.

vaccination in three of four patients (MK, SK and GG). These cells were detected in slightly higher numbers after vaccination in all three patients. Furthermore, in patient RE no T lymphocytes were observed before therapy, whereas after vaccination those T cells accumulated around and partly infiltrated the tumor (Table 5A and Figure 4).

Discussion

There is no hope of curing a metastatic melanoma. Since melanoma is a highly immunogenic tumor, alternative treatment modalities are aimed at the immunological modulation. Previous animal studies suggested that a potent protective immune response can be generated *in vivo* using cytokine gene-modified tumor cells.[5,6] The possible mechanisms by which cytokine-modified tumor cells may function as vaccines have been reviewed elsewhere.[5,6] Active immunotherapy of certain human cancers including malignant melanoma using cytokine gene modification of autologous tumor cells is currently being tested in a number of clinical trials[20,21] including at least

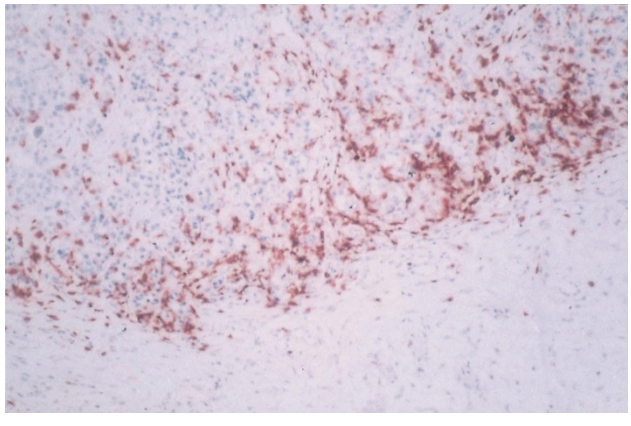

Figure 4 *Immunohistological characterization of tumor infiltrate. Regressing metastasis (RE) removed after final vaccination with autologous IL-12-modified tumor cells stained with anti-CD8 MAb. Note the massive accumulation at the peritumoral areas as well as the infiltration of CD8+ T lymphocytes.*

one other IL-12 trial in melanoma.[22] IL-12 has been shown to mediate tumor regression following intravenous administration in various animal models[8,9,14,15,18,19] and after gene transfer into fibroblasts or tumor cells.[7,12] However, in many models IL-12 produced tumor inhibition but not a cure.[15]

Here we report on the construction of an IL-12 expressing vector for gene transfer into human melanoma cells. Preclinical data demonstrated the bioactivity of the secreted IL-12 upon ballistic gene transfer by the induction of IFN-γ release. Furthermore, IL-12 secretion could be enhanced by magnetic separation of transfected cells. Irradiation led to further increase with detectable IL-12 levels over at least 7 days *in vitro*. IL-12 does not have a direct antiproliferative effect on melanoma cells as judged by the inability of recombinant IL-12, even at high concentrations, to alter tumor cell proliferation (unpublished data). In a subsequent clinical phase I trial using autologous, IL-12 gene-modified tumor cells for treatment, six patients with advanced metastatic melanoma were immunized. This pilot study demonstrates the feasibility and safety as well as the lack of toxicity of such an approach. Although three patients showed stabilization of the disease and one experienced a mixed response for several months, no major clinical response (CR, PR) was achieved. Recently, a phase I evaluation of a dose escalation of intravenous recombinant human IL-12 in 40 cancer patients described one PR and one (transient) CR.[23]

In two out of six patients in our study, immunological monitoring suggested an increase of melanoma-directed lytic clones in the peripheral blood, when the pre- and post-vaccination status were compared. T lymphocytes and tumor cell interaction is HLA class I dependent. Further T cell analysis using HLA-A1 and HLA-A2 presentable peptides in an ELISPOT assay suggests that none of the known melanoma-associated antigens recognized by T cells were detectable. Nevertheless, T cells were shown to be critical for optimal antitumor effects of IL-12.[15] Other research groups using either IL-2-transfected allogeneic tumor cells or autologous tumor cells mixed with IL-2-modified, allogeneic fibroblasts for immunization also had only limited success in detecting specific antitumor T cell reactivity in peripheral blood.[24,25] However, immunological changes connected with IL-12 vaccination were indicated by the development of DTH skin reactivity towards autologous, non-modified tumor cells. Furthermore, CD8 cells infiltrating tumor metastases undergoing regression could be observed, thus indicating that an antitumor immune response had been stimulated. Whether or not the immunological changes observed are solely caused by the IL-12 transfection could not be tested in this initial phase I study. Ethical requirements did not allow a control group with non- or mock-transfected tumor cells at this point.

It has been described that IL-12 can reverse mucosal tolerance upon subcutaneous immunization[26] and induce severe arthritis[27] in mouse models. However, vaccination with IL-12-modified tumor cells did not induce any clinical symptoms or changes indicative of autoimmunity such as an increase of antinuclear or antithyroid antibodies, or of rheumatoid factor (data not shown).

In conclusion, the vaccination with gene-modified tumor cells seems to be tolerated well. Autologous tumor vaccines contain all of the tumor antigens relevant for the individual patient. However, preparation is very labor-, cost- and time-intensive with long latency periods needed to expand the tumor cells *in vitro*. Adjuvant immunization which is more likely to alter the clinical course in tumor patients, in contrast to the set of patients treated in this study, is severely hampered by the necessity of autologous tumor tissue. Therefore, clinical trials using allogeneic tumor cell lines for vaccination are increasingly favored. Vaccination trials using an allogeneic tumor cell vaccine which is well-defined in terms of tumor antigens, secretion of immunosuppressive mediators and expression of HLA molecules allow easier standardization of the immunological detection assays, thus providing an excellent means of evaluating the potential advantages as well as limitations of this new treatment modality in the near future.

Patients and methods

Patient selection

In accordance with the protocol which has been published,[28] accessible melanoma metastases were surgically removed, and melanoma cells were expanded *in vitro* and transduced by ballistic gene transfer (as described below). After magnetic enrichment and irradiation of IL-12 gene-modified tumor cells, patients were immunized s.c. at weekly intervals using multiple aliquots of the cell preparations. Patients were required to have histologically proven metastatic melanoma, adequate hepatic and renal function (bilirubin <3 mg%; serum creatinine <1.5 mg%) and must have had at least one previous unsuccessful systemic treatment including chemotherapy, immunomodulators or a combination of both. A life expectancy of more than 8 weeks was required. Furthermore, patients with any severe cardiac or psychiatric disease or with a concurrent acute infection with hepatitis virus or HIV were excluded. Treatment was carried out at the Department of Dermatology, Virchow Clinic, Berlin. All participants gave informed consent before enrolling in the study, as required by the Institutional Ethical Review Board and in accordance with the Declaration of Helsinki. Patient recruitment started in February 1996 and the study was closed in October 1996. Six patients were enrolled who had all finished at least one chemotherapy regimen which included in four cases an immunomodulator such as IFN or IL-2. Five of six of the patients had metastases in multiple organ sites. Three patients suffered from brain metastases at the start of the therapy. Nevertheless, Karnofsky index before vaccination was above 70 in five of six patients showing at least some DTH reactivity against common recall antigens (Table 1).

Treatment

Within 1 week before vaccination, medical history was taken, and the following baseline studies were performed: a physical examination, hematological testing (hemoglobin, hematocrit, leukocyte, platelet count), blood chemistry panel, antinuclear antibodies, rheumatoid factor, antithyroid antibodies and urine analysis. Blood was also taken for immunological testing. Delayed type hypersensitivity skin tests were performed with the commercially available Multitest Merieux test (Leimen,

Germany) with cell lysates from autologous melanoma cells, and with PBMC before vaccination and at 5-week intervals thereafter. Chest radiograph and computer tomographic scans of brain, chest and abdomen were taken unless previously obtained within the previous 8 weeks. Eligible patients received three vaccinations at weekly intervals. The fourth vaccination was administered in week 6 in accordance with the published protocol.[28] In most instances, the vaccine preparation was split into equal aliquots of about 10^6 cells each and administered s.c. intradermally in close proximity to the regional lymph nodes of each extremity. Comprehensive immunological screening, hematological testing and a crude clinical assessment (physical examination, chest radiograph and ultrasound examination of the abdomen) were repeated during week 5 before the fourth vaccination. A complete clinical and immunological screening comparable to the initial work-up was done during treatment week 10. Vaccinations were continued at monthly intervals thereafter. Patients were followed up until death.

Clinical response and toxicity criteria

Although a clinical response could not be expected and was not the primary aim of this phase I trial, tumor sites were evaluated by physical examinations and scans at 6 to 8 week intervals. Standard definitions of major (complete or partial) objective responses (CR, PR) were used. A minor response (MR) was defined as a 25–50% decrease of lesion lasting at least 1 month, or a more than 50% decrease of lesions lasting less than a month. Stable disease (SD) was defined as less than a 25% change in size with no new lesions developing for 6 weeks. Survival was measured from diagnosis of the first distant metastasis or start of vaccination using gene-modified autologous tumor cells. Adverse effects were recorded using common WHO toxicity criteria.

Preparation of autologous melanoma cells

Tumor specimens were collected from patients with advanced melanoma undergoing procedures either as a part of the diagnostic work-up or for palliative treatment of their disease as described.[29] Solid tumor specimens from lymph nodes, cutis or subcutis were placed immediately after removal into RPMI 1640 (GIBCO, Eggenstein, Germany). Adjunct nonmelanoma containing tissue was removed as completely as possible by scalpel or scissors, and tumors were subsequently cut into pieces. After passing the pieces through a steel mesh with a pore size of 25 mm, the cells were washed twice and cultured in complete RPMI 1640 medium supplemented with 20% fetal calf serum (FCS, Seromed, Berlin, Germany). For vaccination, passages two to 10 were used. When histological and immunocytochemical examinations of cytospin preparations of these cultured cells were performed, they were confirmed to be melanoma cells by a S-100 staining index of >95%.

Maintenance of cell cultures

Newly established human melanoma cell lines of patients were grown in complete medium (RPMI 1640 supplemented with 10% FCS, 2 mm glutamine (GIBCO) and 100 U/ml penicillin/streptomycin (Seromed). Cultures were maintained at 37°C in an atmosphere of 5% CO_2 in air. The NK-sensitive cell line K562, autologous EBV-immortalized B cell lines and autologous melanoma cells, all grown in complete medium, were used as further target cells in the immunological assays. The T cell lines were generated and maintained in T cell medium (RPMI containing 10% pooled human AB serum (Sigma, Deisenhofen, Germany), 25 mm Hepes buffer, 2 mm glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 U/ml IL-2). After 4 weeks of limited dilution (LD) culture, growth of generated T cell lines was further supported by addition of autologous inactivated EBV-immortalized B cell lines. For the LDH release assay, a separate medium consisting of RPMI 1640 without phenol red (Seromed) was used supplemented with 2 mm glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 3% FCS (= medium B).

Immunohistochemical analysis

For immunohistology all tissue samples were rapidly frozen in liquid nitrogen and kept at –80°C. Cryostat sections (4–10 μm) were prepared at –25°C, placed on poly-l-lysine coated slides, dried for 20 min, fixed with ice-cold acetone and used immediately or after storage (up to 5 days) at –20°C. Before staining, the sections were fixed for 10 min in acetone at room temperature and subsequently stained by standard APAAP technique, as previously described.[30] The following antibodies were used for staining MAGE-1,[31] MAGE-3,[32] HMB45 (Dako, Hamburg, Germany) staining the melanosomal protein gp100, T311 detecting tyrosinase,[33] A103 recognizing Melan-A/MART-1[34] and TA99 staining TRP-1[35] (all kindly provided by Drs LJ Old and E Stockert, Ludwig Institute for Cancer Research, New York, NY). For analysis of the cellular infiltrate and intratumoral cells, tissue sections were stained with CD1a (NA1/34; dendritic cells), CD4 (MT310; helper T cells), CD8 (DK25; cytotoxic T cells), CD25 (ACT-1; IL-2R chain) and CD68 (KP1, macrophages) separately (all purchased from Dako). For evaluation of immunohistological staining, a standard evaluation form was designed and read by two independent investigators.

Polymerase chain reaction (PCR)

PCR was carried out with reverse transcribed cDNA generated from all melanoma cell lines as described previously.[36] Briefly, the following primer sequences were used: Tyr-1: TTG GCA GAT TGT CTG TAG CC and Tyr-2: AGG CAT TGT GCA TGC TGC TT, which generate a 284 bp DNA amplificate specific for tyrosinase; Tyr-3: GTC TTT ATG CAA TGG AAC GC and Tyr-4: GCT ATC CCA GTA AGT GGA CT generate a second 207 bp DNA amplificate specific for tyrosinase; MAGE1–3: CTT GCC TCC TCA CAG AG and MAGE1–5: TTG CCG AAG ATC TCA GGA A generate a 407 bp DNA amplificate specific for the MAGE-1 gene; MAGE3–5: TGG AGG ACC AGA GGC CCC C and MAGE3–3: GGA CGA TTA TCA GGA GGC CTG C generate a 714 bp DNA amplificate specific for the MAGE-3 gene; pMEL175: AGAQTCC TGC AGG CTG TGC and pMEL173: CAA TGG GAC AAG AGC AGA generate a 540 bp DNA amplificate specific for the gp100/pMEL17 gene; MART1–5: ACT GCT CAT CGG CTG TTG and MART1–3: TCA GCC ATG TCT CAG GTG generate a 265 bp DNA amplificate specific for the MART-1/Melan-A gene; TRP-15: AAA GGA TTA GTA AAG GGT and TRP-13: CAT TCT GCT TGA AAT AAG generate a 670 bp fragment specific for the TRP-1 gene; TRP-25: CTG GGT GCA GAG TCG GCC and TRP-23:

ATT GGG CCC AAG CAG GCC generate a 300 bp DNA amplificate specific for the TRP-2 gene.

Flow cytometry

Flow cytometric analysis was performed on an EPICS XL (Coulter, Krefeld, Germany) as described.[29] Staining of cell surface markers of melanoma cells was performed by unlabeled antibodies detecting HLA-A,-B,-C, HLA-DR, HLA-DQ, ICAM-1 (all obtained from Immunotech, Hamburg, Germany), and HLA-A2 (clone BB72; a gift from Dr Coulie, University of Louvain, Brussels, Belgium) and the respective isotype control antibodies in combination with a FITC-labeled goat-anti-mouse Ig antibody (Immunotech) and the respective isotype control antibodies (all purchased from Coulter, Krefeld, Germany) for 30 min at 4°C.

Determination of IL-10 and TGF-β1

Immunosuppressive mediators such as IL-10 (Immunotech) and TGF-β1 (Genzyme, Cambridge, MA, USA) released by melanoma cells used for vaccination were measured by quantitative immunoenzyometric kits. Melanoma cells (2×10^5) in 2 ml complete medium were seeded in 24-well plates at 37°C in a 5% CO_2 atmosphere for 24 h. Cytokine levels were determined in the supernatants. The sensitivity was 0.05 ng/ml for TGF-β1 and 5 pg/ml for IL-10, respectively, as indicated by the manufacturers.

Ballistomagnetic gene transfer for expression plasmids into melanoma cells

The two chains coding for the bioactive human IL-12 gene (kindly provided by G Trinchieri, Wistar Institute, Philadelphia, PA, USA) were independently cloned via *Nhe*I (p35) and *Xba*I (p40), respectively, into a eukaryotic expression vector pRc/CMV (Invitrogen, Heidelberg, Germany). Both constructs were entirely sequenced before use as described.[28] For gene transfer, the ballistomagnetic vector system that is a two-step procedure was used as described previously.[29] Ballistomagnetic transfer of expression plasmids into 10^7–3×10^7 cells seeded on to a 10 cm Petri dish was achieved by simultaneous delivery of particles from seven particle carrier membranes which were arranged so as to cover evenly the entire area of the Petri dish. Cell culture medium was removed from the Petri dish immediately before operating the ballistomagnetic vector system. Following ballistomagnetic transfer, cells were immediately resuspended in 5 ml of PBS and transferred on to a high-gradient magnetic separation column (capacity 3×10^7 cells, type AS, Miltenyi, Bergisch Gladbach, Germany) which was prepared according to the supplier's protocol. An aliquot of the cell suspension was kept for reference (unsorted fraction). The cell suspension was passed through the column, followed by a washing step with 3 ml of PBS. The effluent was collected (negative or nonmagnetic fraction). Following removal of the column from the magnetic separator, the retained cells were flushed back to the top of the column. The column was put back into the separator, washed with 3 ml of PBS, and the effluent collected thereafter (wash fraction). Finally, the column was again removed from the separator and eluted with 5 ml of PBS (magnetic fraction). Recovered cells were sedimented at 400 *g* at 4°C for 7 min and resuspended in tissue culture medium. Cells were incubated under culture conditions for 24 h.

IL-12 secretion after gene transfer and irradiation

The following day gene-modified melanoma cells were irradiated with 100 Gy in a small volume. Cells were subsequently detached from flasks by mechanical scraping and ice-cold PBS, washed three times with sterile PBS and finally counted and resuspended with 5×10^6 gene-modified melanoma cells per ml. An aliquot of 10^6 cells was transferred to a single well of a 24-well plate (Nunc, Roskilde, Denmark) for determining cytokine secretion after 24 h, using an IL-12 ELISA (R&D Systems, Minneapolis, MN, USA). Detection range was between 10 and 200 pg/ml according to the manufacturer's instructions. Homodimer formation was tested by using an additional p35 and p40 ELISA (Pharmingen, Hamburg, Germany). The bioactivity of IL-12 containing supernatant was analyzed and confirmed with a bioassay analysing the release of IFN-γ from peripheral blood lymphocytes (PBL) upon contact with cell culture supernatants. PBMC were separated by Ficoll–Hypaque density gradient (Seromed) from two independent healthy donors. PBL were prepared from PBMC after partial depletion of monocytes by adherence to plastic surfaces (45 min, 37°C) and plated in U-bottom microtiter plates (10^6 cells/100 μl RPMI/10% FCS). One hundred microliters of IL-12 containing supernatant were added in duplicates to the PBL and incubated for 18 h (37°C, 5% CO_2). Controls included recombinant human IL-12 (Sigma) at 0.5 and 5.0 ng/ml. Furthermore, each sample was tested in parallel by addition of IL-12 neutralizing goat anti-human antibodies (Sigma) at 2 μg/ml (= 20-fold EC_{50} concentration) with PBL being preincubated with antiserum (30 min, 37°C, 5% CO_2) before addition of the supernatants.

Delayed-type hypersensitivity (DTH)

DTH tests were performed 2 days before the first vaccination and during weeks 6 and 12 of treatment. Two preparations were used each time, one consisting of an autologous melanoma cell lysate and the other consisting of autologous PBMC lysate as a negative control. The melanoma test preparation was prepared from autologous cells of each patient, starting with 4×10^6 suspended, mitomycin-inactivated (45 min, 37°C) primary melanoma cells. After PBS washings, cells were lysed by three cycles of freeze–thawing. Aliquots of 1.3×10^6 cell equivalents were stored in 0.3 ml PBS per tube at –80°C. The autologous PBMC lysate was prepared after separation of leukocytes by Ficoll centrifugation, using otherwise the same procedures as described above for melanoma cells. For DTH testing, 1×10^6 cell equivalents were injected intradermally into the forearm. In parallel, a commercially available recall-DTH test (MultiTest Merieux) was administered on the opposite forearm. A positive skin-test reaction was defined as >5 mm diameter induration after 48 h.

Preparation of autologous lymphocytes

PBMC were obtained after informed consent and isolated from heparinized peripheral blood by Ficoll–Hypaque (Biorad, Berlin, Germany) density centrifugation. Cells were washed twice with PBS, and either resuspended in complete culture medium (see target cells) or cryopreserved in liquid nitrogen in the presence of more than 50% FCS for ELISPOT assay or limited dilution microcultures. EBV-immortalized B cell lines were used as autologous targets for feeder cells supporting T cell growth.

Immortalized B cell lines were generated by a standard method using B95–8 marmoset cell line supernatant containing EBV.[37]

Limiting dilution microcultures

To estimate changes in the frequency of tumor-reactive T cells as well as tumor-specific CTL precursors in the peripheral blood, we used a limiting dilution analysis (LDA) method, described by Coulie, with minor modifications.[29,38] Briefly, cryopreserved PBMC obtained before vaccination and after the fourth vaccination were seeded at limiting dilutions in microcultures of 96-V-bottom microwells (Nunc) with 1×10^4 mitomycin-inactivated autologous melanoma cells in the presence of 20 U/ml IL-2 and 200 μl T cell medium. Cell viability was required to be above 85% by trypan blue exclusion and cell counts were done by two independent investigators. At least 48 microcultures were set up for each dilution of PBMC (20 000, 13 333, 8889, 5926, 3951 and 2634 cells per well). On day 14, the cells were transferred into flat-bottom microwells (Nunc). Microcultures were restimulated on days 7, 14 and 21 with 1×10^4 inactivated melanoma cells per well with 20 U/ml IL-2 in either 100 μl fresh medium/V-bottom well or 200 μl fresh medium/flat-bottom well, respectively. On day 25, microcultures were washed once and resuspended in 200 μl medium B. Three aliquots of 60 μl were transferred into U-bottom microwells (Nunc) to test their lytic activity in a LDH release assay.

Determination of cytotoxicity of responder lymphocytes upon coculture with autologous melanoma cells using the LDH release assay

All microcultures wells were analyzed regarding cytolytic activity using the LDH release assay. The LDH release assay is a colorimetric enzyme release test that showed a good correlation to the radioactive ^{51}Cr release assay.[39] Here, it was used to measure the lytic activity generated in the LDA microcultures against nonadherent target cells (eg the NK-sensitive cell line K562) and autologous melanoma cells. For further T cell line characterization lytic activity was also tested against autologous EBV-immortalized B cells.

The assay was performed using a commercially available detection kit for LDH (Boehringer Mannheim, Mannheim, Germany) which detects released LDH by reduction of a tetrazolium salt (INT) to a water-soluble red formazan salt mediated by NAD+/NADH and lactate/pyruvate. Briefly, effector cells obtained from LDA microculture were washed and split into three aliquots of 60 μl and transferred to 96-well U-bottom microplates (Nunc). Target cells (5×10^3) (autologous melanoma or K562 cells, respectively) in 100 μl per well medium B were added. Plates were incubated for 6 h at 37°C in 5% humified CO_2. After centrifugation at 250 *g* for 7 min, supernatants were transferred to corresponding wells of 96-flat-bottom microwells (Nunc) and 100 μl test kit solution (catalyst and dye solution) was added to each well. After incubation for 30 min at room temperature, the absorbency (A) at 492 nm wavelength was determined using an ELISA reader (Titertek Multiscan MCC/340, Meckenheim, Germany). Medium B served as background control. The specific lysis was calculated as described.[29] Spontaneous release of target cells ranged between 10 and 20% of total release.

Analysis of T cell reactivity in microcultures

All microcultures with an LDH release exceeding the mean spontaneous release from target cells (T) (measured in 12 control wells) by at least 3 s.d. were considered cytolytically positive. The statistical method of precursor cell frequency, 95% confidence intervals, and *P* values indicative of single-hit kinetics were determined by a computer program based on published methods[40] kindly provided by Dr Heeg (University of Munich, Germany).

Statistical analysis

Statistical significance of the data obtained from the cytotoxicity assays was calculated using a SPSS computer package. A modified Wilcoxon signed-rank test and a Mann–Whitney *U* test were used.

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