Intratumoral Injection of DNA Encoding Human Interleukin 12 into Patients with Metastatic Melanoma: Clinical Efficacy

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ABSTRACT

Plasmid DNA encoding human interleukin 12 (IL-12) was produced under GMP conditions and injected into lesions of nine patients with malignant melanoma (stage IV) previously treated with both standard and nonstandard therapies. The treatment was based on efficacy in preclinical studies with melanoma in mice and gray horses. The DNA was applied in cycles, three injections per cycle, for up to seven cycles. Three therapy arms comprised low (2 mg), medium (4 mg), and high (10 to 20 mg) amounts of total DNA. The therapy was well tolerated. Three of nine patients experienced a clinical response: two stable disease and one complete remission. One patient receiving a low dose of DNA experienced a long-lasting stabilization of the disease for more than 3 years, whereas the other two responders received high doses of DNA. All patients but one (patient 9) experienced a transient response at the intratumoral injection site. Immunohistochemical staining of responder sections showed local reduction of angiogenesis and lymphocyte infiltrations. All patients, in particular the clinical and local responders (patients 3, 7, and 8), exhibited an antigen-specific immune response against MAGE-1 and MART-1, which in some cases preexisted. Biopsies of responders showed some increase in IL-12, IP-10, and IFN-γ. Serum levels revealed fluctuations. The results show that intratumoral injection of DNA produced some beneficial clinical effect. DNA encoding a cytokine may be useful as a therapeutic or adjuvant against various human cancers.

OVERVIEW SUMMARY

At present, there is no effective treatment for melanoma in an advanced stage. However, preclinical studies with several animal models have demonstrated that immunotherapy with a plasmid encoding the cytokine IL-12 leads to a pronounced reduction of tumor growth. We evaluated the safety and efficacy of intratumoral application of plasmid DNA encoding IL-12 in a phase I/II clinical study with nine patients with stage IV malignant melanoma. The therapy was well tolerated, with no occurrence of adverse side effects. Application of IL-12 DNA induced clinical responses, one complete remission and two stable diseases, and local responses at the injection site in all except one patient. Four patients exhibited responses at distant metastases. Thus, IL-12 applied as gene medicine to patients shows potential as an antitumor agent.

INTRODUCTION

MALIGNANT MELANOMA is a tumor with increasing incidence. There is currently no cure for advanced disease with distant metastases. Specific or unspecific immunotherapies have been shown to induce tumor regressions in a minority of patients (Haffner et al., 1992; Dummer et al., 1998; Nes-tle et al., 1998; Duran Garcia et al., 1999; Marchand et al., 1999; Stopeck et al., 2001).

The heterodimeric cytokine interleukin 12 (IL-12) consists of two subunits, p40 and p35 (Trinchieri, 1994). IL-12 has been applied as a recombinant protein in cancer patients in various phase I and II studies and found to show some efficacy (Atkins et al., 1997; Bajetta et al., 1998; Motzer et al., 1998; Robert-son et al., 1999). However, its use induced severe toxicity that caused two deaths in a phase II study (Cohen, 1995; Leonard et al., 1997). IL-12 is a key mediator of innate and cellular im-

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munity and improves tumor recognition by direct upregulation of HLA class I, HLA class II, and intercellular adhesion molecule I (ICAM-1) (Yue et al., 1999) on melanoma cells. It enhances tumor destruction by activation of cytotoxic T lymphocytes (CTLs), by priming differentiation of naive CD4+ T cells to helper T type 1 (Th1) cells that can subsequently provide help for tumor-specific CTLs, and by stimulation of natural killer (NK) cells (Kobayashi et al., 1989; Chan et al., 1991; Trinchieri, 1994). Furthermore, IL-12 has been shown to have antiangiogenic properties (Folkman, 1995; Voest et al., 1995; Coughlin et al., 1998; Morini et al., 2003). Some of it is conferred by its downstream mediator interferon γ (IFN-γ) and IFN-inducible protein 10 (IP-10) (Angioliollo et al., 1995).

Preclinical studies have shown that IL-12 applied as plasmid DNA intramuscularly or into tumor lesions was most efficient compared with a number of other cytokines, leading to regression of tumors and preventing the establishment of metastases (Schultz et al., 1999, 2000; Heinzerling et al., 2001, 2002). Injection of plasmid DNA stimulated endogenous IL-12 production by the host, inducing long-lasting expression and amplifying its potency (Schultz et al., 2000). Antiangiogenesis contributes to the antitumor potency (Schultz et al., 1999, Heinzerling et al., 2001, 2002; Morini et al., 2003).

On the basis of these preclinical results on melanomas in mice and gray horses, a human dose escalation trial was approved for nine late-stage malignant melanoma patients, using intratumoral IL-12 DNA therapy.

Here we show that the DNA application, up to high doses, was tolerated well. One patient (P7) showed complete remission of a large lesion, making foot amputation unnecessary, and three other tumors regressed dramatically (in P3, P4, and P8). All but one treated lesion showed some local response. Some patients had regional effects in the draining lymph nodes and four patients showed a response in some distant metastases. Some of the patients showed stable disease during treatment. The antitumor response may be attributable to several effects including IFN-γ production, antiangiogenesis, and lymphocyte infiltration. Thus DNA encoding a cytokine has proved to be a useful anticancer therapeutic.

MATERIALS AND METHODS

Patients

Patients were eligible for the study if they had histologically verified metastasizing stage 4 melanoma with an accessible lesion. Prior therapies are summarized in Table 1.

Plasmid DNA

Cloning of the plasmid DNA has been described previously (Schultz et al., 1999, 2000; Heinzerling et al., 2001). The plasmid contains human IL-12 cDNA under the control of the cytomegalovirus (CMV) immediate-early enhancer/promoter region. DNA fragments encoding the two subunits of IL-12, p35 and p40, were linked by an internal ribosomal entry site (IRES). The plasmid DNA was sequenced before further processing. The DNA, consisting of the covalently closed circular (ccc) form, was prepared by T. Green (Puresyn, Malvern, PA). It was used to transform bacteria (Escherichia coli DH5α) for the production of plasmid DNA by Berna Biotech (Bern, Switzerland) under Good Manufacturing Practice (GMP) conditions. Toxicology tests were performed. Aliquots were prepared by the manufacturer according to the dosing scheme in the protocol, keeping the total volume per injection constant (1 ml in phosphate-buffered saline [PBS]). Expression of both subunits in serum samples was verified after intramuscular injection of plasmid DNA into C57BL/6 mice, using commercially available kits. The level of detection was 5 pg/ml. The level of expression of murine IL-12 DNA (100 μg) injected intramuscularly into mice resulted in about 30 pg of p70 per milliliter of serum (Schultz et al., 1999, 2000), while no ectopically expressed IL-12 was detectable in serum after intratumoral injection. The level of IFN-γ detectable in mice injected intramuscularly with 100 μg of murine IL-12 DNA amounted to 250 pg/ml, corresponding to 2.5 units (100 pg of IFN-γ equals 1 unit). The level of detection was approximately 5 pg/ml for mouse IFN-γ (Schultz et al., 1999, 2000).

Study design

This investigator-driven study was an open-label, single-center phase II/I dose escalation trial. The clinical protocol had been approved by the Swiss authorities: the Swiss Commission for Biological Safety (SKBS), the Swiss Federal Office of Public Health (Swissmedic), and the ethics committee of University Hospital Zurich; and agreed with the Helsinki Declaration.

Plasmid DNA was injected by syringe and needle directly into one selected metastasis with one or two punctures, using ultrasound guidance when necessary. Patients were divided into three groups of three patients each, receiving low, medium, and high doses of DNA. The treatment was performed in cycles. One cycle consisted of three injections at weekly intervals, that is, on days 1, 8, and 15, followed by a resting period of about 8 days. The cycles were repeated at least once and up to six times. The same tumor site was used for administration of all three doses of a cycle. For safety reasons, the first group received two cycles before the next group was initiated. The patient’s general condition was monitored before and after every injection. After two cycles tumor staging was performed. Biopsies were taken before treatment and after two cycles of treatment in all cases, but only eight pairs qualified for evaluation. All patients received a predose of 50 μg of DNA on day –14. The cycles of group 1 comprised 3 × 100 μg, 3 × 200 μg, and 3 × 200 μg for each further cycle. Group 2 received 3 × 250 μg, 3 × 500 μg, and 3 × 500 μg for each further cycle. Group 3 received escalating doses of 250, 500, and 750 μg in the first cycle, 3 × 1000 μg for the second cycle, and 3 × 1000 μg for further cycles.

Tumor measurements

Tumor lesions were selected to allow staging by positron emission tomography (PET), computer tomography (CT), magnetic resonance imaging (MRI), ultrasound (US), or physical examination. All sites of metastatic disease were documented. The tumor was assessed at the beginning of therapy, and then at bimonthly intervals until progression. Clinical response was rated as defined in the study protocol for overall tumor response, with partial response indicating at least a 50% reduction in the tumor masses, stable disease indicating that disease decreased...
less than 50% and increased by no more than 25%, and progressive disease defined as a more than 25% increase in tumor masses or the occurrence of new tumors.

Safety parameters

Patients were monitored for adverse events (AEs) during each visit of the study. Standard laboratory parameters were tested at the beginning and at weekly intervals 24 hr postinjection, including day 1 and after the resting period, for a total of five times (i.e., on days 1, 9, 15, 22, and 28 of each treatment cycle). All patients tested negative for CMV infection. This test was performed as a safety measure, because the plasmid contained the CMV immediate-early promoter, which may be affected by virus infection.

Immunologic parameters

Blood samples were taken on screening days, that is, on days −14, −13, 1, 2, 3, 7, 8, 9, 15, 16, 22, and 28 of each cycle. Serum was frozen and analyzed later by enzyme-linked immunosorbent assay (ELISA) for IL-12, IFN-γ, IL-10, IL-6, IL-2, IL-2R, IP-10, and tumor necrosis factor α (TNF-α). Human S-100 was measured during screening and after two cycles. Serum samples were tested for antibodies directed against MAGE-1 and MART-1 proteins by ELISA. Coating of the plates with recombinant MAGE-1 and MART-1 proteins (Spring Bioscience, Fremont, CA) and the ELISAs were carried out as described by Hoon and co-workers (1995), using various serum dilutions (1:50, 1:200, and 1:1000).

Biopsies

Biopsies for histologic evaluation and immunohistochemistry were taken before treatment and 1 day after the last injection of the second cycle (e.g., on day 44). Biopsy specimens were divided and half of the tissue was snap-frozen, while the other half was fixed in formalin. All biopsies were taken by the investigators and snap-frozen according to a standard procedure. Samples were stored until further use in a designated refrigerator with temperature control.

Immunohistochemistry

Hematoxylin–eosin (H&E) and periodic acid–Schiff (PAS) stainings were performed on paraffin-embedded tissue sections.

### Table 1. Clinical Data on Patients 1–9: Pretreatment

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex, age (years): initials</th>
<th>Diagnosis (Breslow thickness, mm)</th>
<th>Metastases</th>
<th>Previous therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M, 49; S.E.</td>
<td>NM (2.5)</td>
<td>Left thigh i.m., s.c.</td>
<td>LA, X-R, IT (IFN-α, ITR), DC, CH (vinodesine), S</td>
</tr>
<tr>
<td>2</td>
<td>F, 59; S.U.</td>
<td>Unknown primary</td>
<td>Abdominal, iliacal, bone</td>
<td>X-R, CH (temozolomide), IT (IL-2, IFN-α)</td>
</tr>
<tr>
<td>3</td>
<td>F, 58; T.H.</td>
<td>ALM (2.0)</td>
<td>s.c., i.m., LN, mediastinal, pulmonary</td>
<td>LA, CH (temozolomide), IT (IL-2, IFN-α), X-R</td>
</tr>
<tr>
<td>4</td>
<td>M, 67; N.S.</td>
<td>Unknown primary</td>
<td>Multiple bone, brain, s.c., LN, adrenal, renal, duodenal</td>
<td>Neck dissection, X-R LN, S, IT (IFN-α), CH (temozolomide), X-R, gamma knife (brain)</td>
</tr>
<tr>
<td>5</td>
<td>M, 82; Y.F.</td>
<td>NM in SSM (2.7)</td>
<td>s.c., bone, LN, adrenal, peritoneal, thymus</td>
<td>LA, IT (Iscador), S, CH (temozolomide), X-R</td>
</tr>
<tr>
<td>6</td>
<td>M, 59; M.J.</td>
<td>Melanoma (0.65)</td>
<td>Multiple s.c., LN, pleural, mediastinal, peritoneal, liver, spleen</td>
<td>LA, IT (antigen vaccination), X-R, S, CH</td>
</tr>
<tr>
<td>7</td>
<td>F, 66; L.S.</td>
<td>ALM</td>
<td>s.c., bone</td>
<td>S, X-R</td>
</tr>
<tr>
<td>8</td>
<td>F, 44; Y.B.</td>
<td>SSM (0.68)</td>
<td>LN, s.c.</td>
<td>S, LA, IT (IFN-α)</td>
</tr>
<tr>
<td>9</td>
<td>M, 66; H.U.</td>
<td>Unknown primary</td>
<td>Pulmonary, mediastinal LN, adrenal, renal, gall bladder, mesenteric, s.c., i.m.</td>
<td>S, IT (IFN-α, IL-2), CH (temozolomide)</td>
</tr>
</tbody>
</table>

Abbreviations: ALM, acrolentiginous melanoma; CH, chemotherapy; DC, dendritic cell vaccination; F, female; IFN-α, interferon α; IL-2, interleukin 2; IT, immunotherapy; i.m., intramuscular; ITR, isotretinoin; LA, lymphadenectomy; LN, lymph node; M, male; NM, nodular melanoma; S, surgery; s.c., subcutaneous; SSM, superficial spreading melanoma; X-R, radiotherapy.

*Number indicates vertical tumor thickness (mm).
Immunostainings for CD3, CD4, CD8, CD31 and CD56, S100, HMB-45 (for gp100 expression), MAGE-3 (all antibodies from DakoCytomation, Glostrup, Denmark), Melan A (Novocastra, Newcastle, UK), and Ki67 (Immunotech, Marseille, France) were carried out on paraffin-embedded sections.

An anti-DNA antibody assay was performed as described previously (Weber et al., 2001) before and during therapy and proved negative.

Real-time polymerase chain reaction analysis of biopsies

For all samples, total RNA was isolated by the first author in order to limit interpersonal differences due to extraction technique. RNA isolation from biopsies was performed with TRIzol reagent (Invitrogen, Basel, Switzerland) according to the manufacturer’s protocol. Approximately 1 μg of RNA was reverse transcribed, using a 15-mer oligo(dT) primer and avian myeloblastosis virus reverse transcriptase (first-strand cDNA synthesis kit for reverse transcription-polymerase chain reaction [RT-PCR]; Roche Diagnostics, Mannheim, Germany) at 42°C for 1 hr. Complementary DNAs (cDNAs) were then stored at −20°C until further use. Eight matching samples taken before and after therapy qualified and were combined, and are shown below.

Real-time quantitative PCR analysis was conducted on a LightCycler machine (Hoffmann-La Roche, Basel, Switzerland) with extracts of 22 frozen samples and primers specific for the p40 subunit of IL-12 (5’-CTCCCTGACATCTTGTTCA-3’) and the IRES sequence contained in the plasmid DNA (5’-TGCAATCCTTTGGCGAAG-3’).

For analysis of IL-12, IL-10, CD4, CD8, IFN-γ, and IP-10 amplification primer mixes for RT-PCR were used (Search-LC, Heidelberg, Germany) according to the manufacturer’s recommendations with the standards provided.

RESULTS

Tumor response of patients

Nine patients with late-stage malignant melanoma were recruited, most of them having numerous metastases. One lesion per patient was selected for injection with doses of plasmid DNA as described in the protocol and Materials and Methods, in a constant volume (1 ml). The DNA was produced under GMP conditions and sequenced, and expression of p35 and p40 was verified after transfection of human embryonic kidney (HEK) 293 cells, by immunostaining and ELISA of the supernatant. All patients received a predose of 50 μg of DNA 14 days (one patient, 7 days) before the therapy cycles were initiated, because of previous reports on the toxicity of initial high doses of recombinant IL-12 protein (Cohen, 1995; Leonard et al., 1997). The treatment and status of all patients before IL-12 DNA therapy and after treatment have been summarized (see Tables 1 and 2). The first group of three patients received low doses for each cycle. They received two to four cycles with 0.95 to 2.15 mg of DNA in total. Two of the three patients showed local responses with reduction in tumor size (Table 2). One patient, P3, showed remission of a large axillary tumor (about 8 cm in diameter) that regressed to less than half that size, leading to a significant improvement of quality of life, due to its specific location.

The second therapy arm with medium dose was started after all three patients of the first group had received two cycles and showed no side effects. In the second group two cycles per patient were applied with 2.3 mg per patient in total. With this low dose one patient, P4, showed strong tumor regression (5900 to 229 mm³) and regional stable disease at the draining lymph node (Table 2) (see below [Fig. 2c and d], ultrasound measurements before and after therapy).

The third group received the highest doses with dose escalation for up to seven cycles: patients 7, 8, and 9 received 10.55, 19.55, and 4.55 mg of DNA in total. Even at the highest dose applied over 10 months, no severe side effects were observed. P7 had a large lesion on the foot, about 5 cm in diameter, affecting the bone, with the only therapeutic option being foot amputation. This lesion showed complete remission after three of four cycles with 7.55 mg of the final 10.55 mg of DNA (see below, and Fig. 2). P8 had complete local regression of the treated axillary lesion and stable disease over 10 months of therapy and several more months thereafter. During therapy she developed a new nodule, which remained stable. Her health deteriorated 9 months after termination of the IL-12 DNA therapy and she died after an additional 3 months. Five patients showed considerable tumor regression at the injection site (see Table 2: P3, P4, P6, P7, and P8), with two complete responses, two partial responses, and one stable disease.

We were surprised to note that P3 did well at a low dose of 2.15 mg of DNA, which caused a dramatic local regression of a large axillary tumor, and P4 showed strong tumor regression with 2.3 mg of total DNA. The two largest tumors (P3 and P7) showed the greatest regression.

It was unexpected that four patients (P3, P4, P6, and P8) showed regional effects in the draining lymph nodes, indicating that the therapy was not locally restricted, and four patients responded at some distant metastases (see data for P3, P4, P6, and P8 in Table 2: +/–, indicating some nodes responded whereas others were progressive). One patient (P6) showed a partial response at a regional lymph node without regression of the injected tumor nodule. One patient (P4) also exhibited vitiligo at the forehead, neck, both hands, and one foot after injection of a supraclavicular tumor lesion, indicating an immunological response against melanocytes distant from the site of therapy, proving some systemic response.

Serum cytokine profiles by ELISA and real-time PCR of biopsies

An important question about the therapeutic effect of the locally applied IL-12 DNA concerned the increase in IL-12 and IFN-γ levels either in the serum or locally at the site of injection. For this purpose, serum samples from all nine patients taken at the indicated time points during therapy were tested by ELISA. The data of four responders and three nonresponders are shown in Fig. 1A. The results suggest some increase in IL-12 production in treated individuals, with fluctuations (up to 90 pg of IL-12 per milliliter) not directly correlating with individual injections. IFN-γ levels were high at individual measure-
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex, age (years); initials</th>
<th>Site of injection, dates</th>
<th>Duration (months)</th>
<th>Dose arm</th>
<th>No. of cycles</th>
<th>Dose (mg)</th>
<th>Inj. site resp</th>
<th>Loc resp</th>
<th>Reg resp</th>
<th>Dist resp</th>
<th>Total resp</th>
<th>R/NR</th>
<th>Status</th>
<th>WHO grade (1)</th>
<th>Adverse events, Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M, 49; S.E.</td>
<td>Left thigh, 11/00–3/01</td>
<td>3</td>
<td>Low</td>
<td>3</td>
<td>1.55</td>
<td>Yes</td>
<td>SD</td>
<td>n.a.</td>
<td>n.a.</td>
<td>SD (+)</td>
<td>Alive, 3 years and 7 months</td>
<td>Erythema at injection site; Flattened further therapy; Infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F, 59; S.U.</td>
<td>Left iliac, 2/01–4/01</td>
<td>2</td>
<td>Low</td>
<td>2</td>
<td>0.95</td>
<td>Yes</td>
<td>PD</td>
<td>PD</td>
<td>PD</td>
<td>PD —</td>
<td>Died, 3 months</td>
<td>Hair loss, swollen leg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F, 58; T.H.</td>
<td>Left axilla, 2/01–7/01</td>
<td>4</td>
<td>Low</td>
<td>4</td>
<td>2.15</td>
<td>Yes</td>
<td>PR</td>
<td>SD (+/-)</td>
<td>PD</td>
<td>Died, 8 months</td>
<td>Local erythema</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M, 67; N.S.</td>
<td>Supraclavicular, right, 6/01–8/01</td>
<td>2</td>
<td>Med</td>
<td>2</td>
<td>2.3</td>
<td>Yes</td>
<td>PR</td>
<td>SD (+/-)</td>
<td>PD</td>
<td>Died, 6.5 months</td>
<td>Vitiligo (forehead, neck, hands, and foot)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M, 82; V.F.</td>
<td>Pectoral, supraclavicular; 6/01–8/01</td>
<td>2</td>
<td>Med</td>
<td>2</td>
<td>2.3</td>
<td>Yes</td>
<td>PD</td>
<td>PD</td>
<td>PD</td>
<td>PD —</td>
<td>Died, 7 months</td>
<td>Pain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M, 59; M.J.</td>
<td>Right axilla, 9/01–11/01</td>
<td>2</td>
<td>Med</td>
<td>2</td>
<td>2.3</td>
<td>Yes</td>
<td>SD</td>
<td>PR (+/-)</td>
<td>PD</td>
<td>Died, 3 months</td>
<td>Pain, local erythema</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>F, 66; L.S.</td>
<td>Left foot, 10/01–3/02</td>
<td>4</td>
<td>High</td>
<td>4</td>
<td>10.55</td>
<td>Yes</td>
<td>CR</td>
<td>n.a.</td>
<td>n.a.</td>
<td>CR +++++</td>
<td>Alive, 2 years and 8 months</td>
<td>Reversible hair loss, pain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>F, 44; B.Y.</td>
<td>Left axilla, 12/01–9/02</td>
<td>10</td>
<td>High</td>
<td>7</td>
<td>19.55</td>
<td>Yes</td>
<td>CR</td>
<td>PR</td>
<td>SD (+/-)</td>
<td>Died, 22 months</td>
<td>Reversible hair loss, swelling at injection site</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>M, 66; U.H.</td>
<td>Pectoral, supraclavicular; 12/01–3/02</td>
<td>2</td>
<td>High</td>
<td>2</td>
<td>4.55</td>
<td>No</td>
<td>PD</td>
<td>PD</td>
<td>PD —</td>
<td>Died, 9 months</td>
<td>Itching, pain at injection site</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Sex: M, male; F, female; letter code; age, given in years at beginning of therapy with IL-12 DNA. Site of injection: dates give months and year of beginning and end of therapy. Duration: duration of therapy in months; Dose arm: treatment arm with low, medium, or high dose. No. of cycles: Each cycle corresponds to three weekly injections, 1-week break, and always a pretreatment at the beginning of therapy (2 weeks before). Dose: total dose (mg). Inj site resp: response at injection site (see Remarks). Loc resp: local response; SD, stable disease; PD, progressive disease; PR, partial response; CR, complete response; n.a., not applicable; (+/-), some nodes responded or were stable, whereas others were progressive. Reg resp: regional response, mainly in draining lymph nodes. Dist resp: response at sites other than injected lesion. Total resp: total clinical response of the patient during therapy. R/NR: responder/nonresponder. Status: gives survival time after the beginning of therapy, updated until 6/04. Adverse events: according to WHO grading. Remarks: MRI, magnetic resonance imaging; US, ultrasound; PET, positron emission tomography.
A  Responders

Non-responders

FIG. 1.
ments (up to 300 U of IFN-γ per milliliter), but data correlating IFN-γ with individual injections were also inconclusive. P7, who was a good responder and the only Asian patient, showed almost no IFN-γ levels. ELISAs were also performed on sera for IL-2, IL-2 receptor (IL-2R), IL-10, and IL-6. In none of these cases was a significant increase in serum levels detected (data not shown).

Cytokine levels were also determined in local biopsies of the lesions before and after therapy in selected patients, each receiving two cycles of DNA. Biopsies were taken 24 hr after the last injection. The samples were analyzed by real-time quantitative PCR. For that purpose mRNA was extracted and reverse transcribed. The data of four individual patients (two responders and two nonresponders) reveal a more pronounced increase

**FIG. 1.** Systemic and local cytokine levels during treatment. (A) Serum IFN-γ and IL-12 concentrations of responder and nonresponder patients. Time points of injections and dose of DNA (micrograms) are indicated. Day 0 was the day of the first therapeutic DNA injection. Normal reference level for IFN-γ is 0.1 U/ml or 10 pg/ml and <7.8 pg/ml for IL-12. (B) RT-PCR analyses by real-time PCR of mRNA from tumor biopsies before (open columns) and after (solid columns) two cycles of treatment with IL-12 DNA. The primers were selected to identify the indicated mRNA encoding CD4, CD8, or the cytokines shown. (C) Survival time of individual patients after the beginning of therapy. The gray portions of the columns indicate the duration of treatment; the patterned portions indicate survival after the end of therapy.
in IL-12, IFN-γ, and IP-10, a downstream effector of IL-12, in responders (P3 and P8) whereas in the tumors of nonresponders IL-10 expression appears to increase or remain high during therapy (Fig. 1B).

A survival time plot shows that two patients are still alive; after beginning of therapy one patient lived longer than 3 years; two others longer than 2 years; and four patients lived for 6 to 9 months (Fig. 1C).

**Tumor assessment and histology**

Tumor size was measured with calipers or by ultrasound (US), CT scan, or PET and/or MRI (see Table 2). P7 showed

![Image](image_url)

**FIG. 2.** Effect of IL-12 DNA treatment on the development of melanoma metastases. (a and b) MRI scan (left) and PET scan (right) of a histologically verified melanoma metastasis of the foot (a) before IL-12 DNA treatment of P7 and (b) after three of four cycles of intratumoral injections with IL-12 DNA (corresponding to 7.55 of 10.55 mg). (c and d) P4, with a large supraclavicular tumor, was analyzed by ultrasound before (c) and after (d) two cycles of therapy with 2.3 mg; a strong reduction in tumor volume (5900 to 229 mm³) occurred. The dotted lines indicate the greatest width and perpendicular diameter of the tumor.
complete regression of the tumor on the foot, which was verified by MRI and PET after three of four cycles (intratumoral injections of 7.55 of 10.55 mg of DNA in total) (Fig. 2a and b). One patient (P4) showed a strong reduction in tumor size after two cycles comprising six injections with 2.3 mg of DNA in total, as evidenced by ultrasound analysis of the tumor before and after treatment (Fig. 2c and d; see white dotted lines). The tumor volume decreased from 5900 to 229 mm³.

Tumor biopsies of seven patients (all except P2 and P6) were taken before and after therapy and sections were analyzed by immunohistochemistry (Figs. 3 and 4). Histological features of the tumor of P7 (responder) before therapy included large epithelioid cells with a bizarre morphology of nuclei (Fig. 3A, panel a) and vascularity of the tumor as determined by anti-CD31 staining (Fig. 3A, panel b). After therapy P7 showed pronounced fibrosis and scar formation at the injection site (H&E staining and deposits of melanin in macrophages and between collagen bundles (Fig. 3A, panel c, arrow). Staining of a parallel section with CD31-specific antibodies revealed a reduction in vascularity (Fig. 3A, panel d).

Immunohistochemical analyses of biopsies taken after treatment from two nonresponders (P5 and P9) revealed either peritumoral infiltration of CD4⁺ and CD8⁺ T lymphocytes (Fig. 3B, panels a and b) with focal invasion of a few CD8⁺ T lymphocytes (Fig. 3B, panel a, inset) or showed no detectable infiltration of CD4⁺ or CD8⁺ T lymphocytes (Fig. 3B, panels c and d).

Before therapy, P8 showed abundant blood vessels in and around the tumor (Fig. 4a). During therapy necrosis near the injection site occurred (Fig. 4b, circled portion) and a dustlike distribution of Ki-67-positive nuclear debris was clearly visible (Fig. 4c, circled portion). Before therapy predominantly CD4⁺ helper T cells (Fig. 4d, arrow) were detected in a perivascular localization. Treatment resulted in intratumoral accumulation of CD4⁺ and CD8⁺ T lymphocytes in P8 (Fig. 4d, insets, arrows). Moreover, the therapy induced morphological features of disturbed angiogenesis with partially destroyed blood vessels as shown by H&E staining (Fig. 4e, arrow) and anti-CD31 staining (Fig. 4f and g). Aggregates of erythrocytes after therapy are shown at higher magnification in the inset of Fig. 4e (arrow). In the proximity of thrombosed vessels there was perivascular tumor necrosis and fibrosis (Fig. 4e and g, arrows). Taken together, a pronounced fibrotic reaction with diminished vascularization similar to the features noticed in mice and gray horses was seen (Heinzerling et al., 2001, 2002). No CD56⁺ natural killer cells were detected.

**Tumor antigen-specific immune response**

To analyze whether IL-12 DNA therapy induced some immune response against melanoma-associated tumor antigens, diluted (1:200) serum samples of all patients taken before, after two cycles, and at the end of therapy were analyzed by ELISA for the presence of antibodies against MAGE-1 and MART-1. It was surprising that responders P3, P7, and P8 exhibited higher serum concentrations of tumor-associated antibodies, which in some cases preexisted, than did nonresponders P2, P5, and P9 (Fig. 5).

**Safety parameters**

Intratumoral IL-12 DNA therapy was extremely well tolerated (Table 2). None of the nine patients had a presumably therapy-related AE higher than World Health Organization grade 1. Some patients noticed tension (six patients), erythema (three patients), or other reactions (two patients) at the injection site. There was no discontinuation of treatment due to therapy-related side effects. Some of the patients developed some fatigue, fever, loss of weight or hair, or nausea. Side effects included mild influenza-like symptoms (three patients) and, surprisingly, pain at the sites of noninjected metastases (four patients).

A safety parameter of concern was the presence of anti-DNA antibodies, which may arise as a consequence of DNA-based therapy. These were screened for in the sera of all patients before and during therapy and were negative (data not shown). This is in agreement with results obtained after DNA vaccination of four patients infected with human immunodeficiency virus (HIV) (Weber et al., 2001).

**DISCUSSION**

The present study describes clinical effects of antitumor therapy with plasmid DNA encoding IL-12 in late-stage melanoma patients by direct application of the DNA into one lesion per patient (Table 2). The therapy proved to be well tolerated with no significant side effects, which allowed treatment on an outpatient basis. Up to almost 20 mg of DNA (to our knowledge, the highest dose ever applied to a human) was administered to one patient (P8) over a period of 10 months with minor side effects, such as mild flulike symptoms. This patient died about 1 year after termination of therapy, succumbing to numerous metastases. Intratumoral injection resulted in local reactions at the injection site in all but one of the patients, and in significant local tumor reduction in five patients. The two largest tumors showed the most striking response (see data for P3 and P7). This local response rate is higher than in previous studies using intratumoral application of autologous virally transduced cells expressing IL-12 (Lotze et al., 1997; Sun et al., 1998; Kang et al., 2001). In the present study two large tumors responded strikingly well, one with complete remission (P7), preventing a foot amputation, and one with partial remission (P3) of a large axillary tumor, which improved quality of life significantly. Furthermore, two patients (P4 and P8) showed good local tumor regression either at a low total DNA dose or after only three of seven cycles. It should be noted that the therapy gave rise to some regional lymph node responses in four cases and even to responses in some distant metastases (four cases). A long-distance effect included vitiligo (P4) on the forehead, neck, both hands, and one foot after injection at the chest. Several patients lived for more than 2 or 3 years after the beginning of IL-12 DNA therapy. Thus, we demonstrate here, for naked DNA, local as well as distant antitumor effects, two disease stabilizations, and one complete remission. The preclinical studies proved that the antitumor effects we have observed in these patients are indeed due to the IL-12 insert: the preclinical studies with the IL-12 insert had shown greatly enhanced efficacy when compared with empty plasmid or plasmid with the IL-12 insert in the reverse orientation (Heinzerling et al., 2001). In this clinical study, for ethical and financial reasons no control arm using nonencoding plasmid DNA was used.

Serum levels of IL-12 and IFN-γ fluctuated, with no correlation between injections and response indicated (Fig. 1A). However, biopsies showed some therapy-related effects. Individual samples of responders had more IL-12, IP-10, and IFN-
FIG. 3.  (A) Histopathological analysis of P7 before (a and b) and after (c and d) four treatment cycles. (a) Histological features before therapy. The tumor consists of large epithelioid cells with bizarre nuclei (H&E staining). (b) Vascularization of the tumor before treatment (anti-CD31 staining). (c) Marked fibrosis after treatment with numerous melanophages (arrow) (H&E staining). (d) Anti-CD31 staining after treatment reveals reduction of vascularization and regression of tumor cells compared with (b). Scale bars: 10 μm. (B) Biopsies of nonresponders (P5 and P9) were analyzed immunohistochemically for CD4+ and CD8+ T cell infiltration after two cycles of treatment, showing either peritumoral CD4+ and CD8+ T cell infiltrates with focal invasion of a few CD8+ T cells (a and b) or no infiltrates (c and d). Scale bars: 10 μm.
FIG. 4. Histological evaluation of P8 before therapy (a and d) and after seven cycles of therapy (b, c, e–g, and insets of d). (a) H&E staining of epithelioid tumor cells. (b) Necrosis (circled area) occurring at the injection site (H&E staining), with (c) dustlike distribution of Ki67-positive nuclear debris. (d) Presence of reactive CD4+ T lymphocytes before therapy in the proximity of blood vessels and peritumoral (arrow). CD8+ T lymphocytes were not detectable (not shown). After therapy both CD4+ and CD8+ T lymphocytes were found to have invaded the remaining tumor nests (see insets, arrows). (e) Clotted blood vessels (arrows) with perivascular fibrosis between tumor cell formations (H&E staining; inset shows a clotted blood vessel at 2-fold higher magnification). (f) Immunohistochemistry for CD31 after treatment shows perivascular tumor cell necrosis with some inflammatory mononuclear infiltrates (circled area). (g) Magnification (2-fold) of lower central part of (f), revealing alterations of the blood vessels, including changing calibers (arrows). Scale bars: 10 μm.
The immunological responses of patients against melanoma-associated antigens, such as MAGE-1 and MART-1, were informative because the high-dose group with two responders showed antibody titers—but therapy unrelated. Only one patient (P3) exhibited a transient therapy-related antibody response. IL-12 DNA may have an adjuvant effect for an immune response against the tumors.

Other parameters we tested included anti-DNA antibodies as an indicator of a potential autoimmune reaction against the DNA; these were, however, undetectable (W. Bossart, data not shown). In a previous 2-year follow-up study, autoimmune response against HIV-plasmid DNA was also undetectable (Weber et al., 2001).

Furthermore, recombinant IL-12 had caused the death of 2 of 12 patients after intravenous application in a phase II trial (Cohen, 1995). The recombinant protein had only a short-life (~10 hr) in the body. In addition, recombinant proteins are notorious for misfolding. Primate studies indicated that initial high IFN-γ levels caused severe toxicity. Therefore, a predose was applied in the present study. IFN-γ levels were low and did not induce toxic side effects. None of the previously reported problems arose here such as lymphopenia, neutropenia, increases in transaminases, fever, nausea, vomiting, or stomatitis (Atkins et al., 1997).

Plasmid DNA has been used so far with limited success as a vaccine encoding viral antigens or tumor-associated antigens (Schultz et al., 2000), for example, prostate-specific membrane antigen (Mincheff et al., 2000) or HIV envelope glycoprotein (Weber et al., 2001).

In our own preclinical studies DNA encoding tumor-associated antigens (e.g., gp100), was much less efficient than DNA encoding IL-12 (Schultz et al., 1999). Also, CpG oligonucleotides were rather ineffective, but only a few have been selected for testing so far (Schultz et al., 1999). DNA as a vaccine has been modified by using DNA prime and peptide boost regimens. Peptides may be replaced by larger virus-like structures or recombinant viruses (Amara et al., 2001). Naked DNA encoding vascular endothelial growth factor (VEGF) (Baumgartner et al., 1998; Isner et al., 1998; Losordo et al., 1998) has shown limited success with induction of angiogenesis in humans.

The results here should be extended to more patients and other tumor types, and IL-12 DNA should be combined with other therapeutics. Also, a clinical study should be designed to test whether establishment of metastases can be prevented. This was the most unexpected and unusual property of IL-12 DNA in our preclinical studies. Thus, it may prove valuable as a therapeutic antitumor as well as prophylactic measure against various cancers or metastases in future.
IL-12 DNA IN MELANOMA PATIENTS

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NOTE ADDED IN PROOF

P1 and P7 are still alive.

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