Novel Jet-Injection Technology for Nonviral Intratumoral Gene Transfer in Patients with Melanoma and Breast Cancer

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Abstract

Purpose: This phase I clinical trial evaluated safety, feasibility, and efficiency of nonviral intratumoral jet-injection gene transfer in patients with skin metastases from melanoma and breast cancer.

Experimental Design: Seventeen patients were enrolled. The patients received five jet injections with a total dose of 0.05 mg β-galactosidase (LacZ)-expressing plasmid DNA (pCMVβ) into a single cutaneous lesion. Clinical and laboratory safety monitoring were done. Systemic plasmid clearance was monitored by quantitative real-time PCR of blood samples throughout the study. All lesions were resected after 2 to 6 days. Intratumoral plasmid DNA load, DNA distribution, and LacZ expression was analyzed by quantitative real-time PCR, quantitative reverse transcription-PCR, Western blot, immunohistochemistry, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside staining.

Results: Jet injection of plasmid DNA was safely done in all patients. No serious side effects were observed. Thirty minutes after jet injection, peak plasmid DNA levels were detected in the blood followed by rapid decline and clearance. Plasmid DNA and LacZ mRNA and protein expression were detected in all treated lesions. Quantitative analysis revealed a correlation of plasmid DNA load and LacZ-mRNA expression confirmed by Western blot. Immunohistochemistry and 5-bromo-4-chloro-3-indolyl-β-D-galactoside staining showed LacZ-protein throughout the tumor. Transfected tumor areas were found close and distant to the jet-injection site with varying levels of DNA load and transgene expression.

Conclusion: Intratumoral jet injection of plasmid DNA led to efficient LacZ reporter gene expression in all patients. No side effects were experienced, supporting safety and applicability of this novel nonviral approach. A next step with a therapeutic gene product should determine antitumor efficacy of jet-injection gene transfer.

Safety and efficiency of gene transfer are major challenges for successful gene therapy. Although viral vectors are very efficient for gene transfer, their use is still limited by safety concerns (1, 2). For clinical applications, naked DNA is becoming more important due to its safety profile, easy preparation procedures, and moderate costs (3). As an alternative to viral or liposomal technologies, 18% of all gene therapy trials are currently based on naked DNA gene transfer.5 It was shown that simple needle injection of naked plasmid DNA leads to transgene expression (4, 5). Particularly, for the improvement of local in vivo delivery of naked DNA, different physical procedures are employed including in vivo electroporation, sonoporation, ballistic transfer, or jet injection (6–17).

We focused on the development of jet injection for in vivo gene transfer using naked DNA in small volumes and at small amounts. This technology is based on jets of high velocity that deeply penetrate skin and underlying tissues, leading to efficient transfection (18). We and others showed the effectiveness of in vivo gene transfer for β-galactosidase (LacZ) and green fluorescence protein reporter gene constructs in different preclinical tumor models (15, 18–20). Qualitative and quantitative analysis of jet-injected tumors revealed efficient gene expression and provided data on plasmid biodistribution and systemic clearance (21). Therapeutic in vivo jet-injection transfer of the cytosine deaminase suicide gene or the human

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5 http://www.wiley.co.uk/genmed
tumor necrosis factor-alpha gene showed significant tumor growth inhibition (22, 23).

Based on these strong preclinical data, we conducted a phase I gene transfer trial using jet injection for intratumoral application of small amounts of plasmid DNA. The goals of this trial were evaluation of safety and efficacy of the nonviral jet-injection gene transfer in patients with metastatic melanoma or breast cancer. Here, we report the results of a comprehensive safety evaluation, including quantitative analysis of plasmid DNA distribution and systemic clearance in the blood. We evaluated gene transfer efficiency by analyzing LacZ expression quantitatively and qualitatively at the mRNA and protein levels. Furthermore, detailed data on intratumoral distribution of plasmid DNA and LacZ-protein are provided.

**Patients and Methods**

**Objectives.** The phase I trial evaluated safety and feasibility of the nonviral jet-injection transfer of the LacZ-expressing naked reporter plasmid DNA in skin metastases of breast cancer and melanoma. Levels of transgene expression, plasmid biodistribution within the tumor, and potential plasmid dissemination were analyzed quantitatively and qualitatively. This study was done as a marker study for evaluation of safety and efficiency in cancer gene therapy.

**Study approval.** The trial is registered at the German Clinical Gene Transfer Trial Database6 and was approved by the local ethics committee of the Charité-Universitätsmedizin Berlin and by the Federal Commission of Somatic Gene Therapy at the Bundesärztekammer (German Medical Association). The study was conducted in accordance to the principles of the International Conference on Harmonization of Good Clinical Practice guidelines and the Declaration of Helsinki.

**Managerial Impact**

In this clinical gene transfer trial, we showed that the needle-free, low-volume jet injection of small amounts of naked plasmid DNA is successfully and safely employed for nonviral gene transfer. It represents a feasible and clinically applicable alternative to other established physical transfer technologies. The advantage of this gene transfer technology is determined by deep penetration of DNA and improved intratumoral dispersion. The results of this gene transfer trial support its great potential for therapeutic applications to locally treat accessible metastases from breast cancer or melanoma. Thus, this gene transfer can be applied for improved control of tumors or metastases from different solid tumor entities within the concept of multimodal tumor therapy. In addition, jet injection might also be employed in the context of other cancer gene therapy approaches, such as DNA vaccination, immunogene therapy, or gene suppression strategies using small interfering RNA/short hairpin RNA, as indicated by preclinical studies. Therefore, jet injection provides broader prospects of different clinical applications and can be easily adapted to such specific requirements. In continuation of this study, we will initiate a further clinical trial for the jet-injection transfer of therapeutic genes into tumor lesions of melanoma patients.

**Reported plasmid DNA.** The *Escherichia coli* LacZ-encoding plasmid pCMVβ (Clontech), in which the CMV promoter controls the constitutive LacZ expression, was obtained and tested by PlasmidFactory according to the good manufacturing practice guidelines. The preparations were free of adventitious bacteria and endotoxin. The plasmid concentration was adjusted to 1 μg/μl in 0.9% sodium chloride. Plasmid identity was confirmed by DNA sequencing; plasmid topology was ensured by capillary gel electrophoresis.

**Study design.** Patients with skin metastases from breast cancer or melanoma received five injections of naked plasmid DNA into the metastatic lesion (Figs. 1A and 2A and B). Patients received a total DNA dose of 50 μg by applying a volume of 10 μL plasmid DNA (1 μg/μL) for each jet injection. The treated lesion was removed 2 to 6 days after jet injection (Table 1). Surgery was done according to the initially planned oncologic procedure (e.g., resection of skin metastases in recurrent breast cancer, resection, and/or isolated limb perfusion in melanoma patients), which was independent from the study. Blood samples were collected before and 0.5, 3, 6, 24, 48, and 72 h and 4 weeks after jet injection for analysis of clinical laboratory variables and for quantitative analysis of plasmid DNA dissemination and clearance.

**Eligibility criteria.** Patients ages >18 years with a Karnofsky performance score ≥60 and with surgically accessible, histologically confirmed in-transit metastases from melanoma or skin metastases from breast cancer were enrolled (Table 1). Patients were required to have adequate bone marrow, liver, and renal functions. Patients with a history of severe or medically treated allergy were excluded. Women, who were pregnant or at risk of pregnancy or breast-feeding, were excluded. All patients were able to understand the informed consent form, which was explained individually to each patient. All patients signed the form indicating their awareness of the investigational nature of the gene transfer study.

**Clinical patient evaluation/toxicity and safety evaluation.** Careful patient monitoring was applied throughout the study. Clinical evaluation, including medical history and general physical examination, was done at baseline and defined intervals after jet injection. Patients were monitored for adverse events according to the National Cancer Institute common toxicity criteria during each visit. The treated lesion was photo-documented before and after treatment. Standard laboratory variables and vital signs were tested before jet injection and 0.5, 3, 6, 24, 48, and 72 h and 4 weeks after jet injection. Laboratory testing included complete blood count, platelets, prothrombin time, partial thromboplastin time, electrolytes, creatinine, bilirubin, transaminases, glucose, and C-reactive protein. In addition, blood was taken at each interval for quantitative analysis of plasmid dissemination and clearance.

**Surgical specimens and pathology.** After complete resection, jet-injected specimens were divided and fixed in formalin (for paraffin-embedded tissue) and shock frozen in liquid nitrogen (for cryosections). Serial paraffin sections were independently evaluated for tumor histopathology and possible jet injection-associated alterations by two experienced pathologists. Sections (7 μm) from shock-frozen specimens were used for plasmid DNA, mRNA, and protein analyses, for detection of LacZ expression by immunohistochemistry, and for detection of functional LacZ by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) staining (Fig. 1B).

**DNA isolation and quantitative real-time PCR.** For detection of pCMVβ plasmid DNA, cellular DNA was isolated from serial cryosections (20 μm) and from full blood samples using Trizol (Invitrogen) or Jetquick Blood Mini Kit (Genomed). The recovery rate for plasmid preparations was ~30%, which was considered in all quantitation calculations.

Real-time PCR (95°C for 30 s; 45 cycles 95°C for 10 s, 62°C for 10 s, and 72°C for 10 s) was done using 250 ng cellular DNA in the LightCycler reaction (LightCycler DNA Master Hybridization Probes kit; Roche Diagnostics) in duplicate per sample. For the pCMVβ plasmid, a 125-bp ampiclon (forward primer: 5′-AAGCAGAAAAAGAGACCTCACCGATG-3′, FITC-labeled probe: 5′-CGGTCTGGGAGCCATTTGTG-3′-FITC,
LCRed640-labeled-probe: LCRed640-5'-GGACACCAGCAAGGAGCTGCT-3', and reverse primer: 5'-GTAAAACGACGGGATCGC-3') was produced (syntheses of primers: BioTeZ; syntheses of probes: TIB MOLBIOL).

Plasmid concentration was calculated using a spiked standard curve of serial dilutions of the pCMVβ plasmid (ranging from 12 fg to 25 ng in duplicate) in 250 ng cellular DNA (isolated from nontransduced SW480 human colon carcinoma cells). Quality of the PCR products was evaluated by the corresponding melting curves for each run.

**RNA isolation and quantitative real-time reverse transcription-PCR.** Total RNA from serial cryosections was isolated by Trizol and treated with RNase-free DNase I (Roche Diagnostics). Reverse transcriptase reaction was done using 50 ng total RNA (MuLV Reverse Transcriptase; Applied Biosystems).

Real-time PCR was done as described above. Expression of the LacZ gene was determined in duplicate per sample. For LacZ, a 102-bp amplicon (forward primer: 5'-CCGTTGATGTTGAAGTGGC-3', FITC-labeled probe: 5'-GGGCGGATTCGCGAAGTGGC-3', LCRed640-labeled-probe: LCRed640-5'-CAGCCTGGGCGAGTGGAGCAGA-3', and reverse primer: 5'-CTAATCGAGCAGCTTACCC-3') was produced (syntheses of primers: BioTeZ; syntheses of probes: TIB MOLBIOL). LacZ expression (mean of duplicates) was calculated as relative fold LacZ-mRNA expression compared with the calibrator cell line. Quality of quantitative reverse transcription-PCR (qRT-PCR) was evaluated by the corresponding melting curves.

**Protein isolation and Western blot.** Total protein from cryosectioned tumors was isolated using Trizol. After quantification using BCA Protein Assay (Perbio Science), 30 μg protein was used for Western blot. The membrane was subjected to LacZ-protein (118 kDa) detection using a mouse anti-β-galactosidase antibody (dilution 1:1,000; Cell Signaling). Equal loading was confirmed by using glyceraldehyde 3-phosphate dehydrogenase-specific antibody (1:500; Santa Cruz Biotechnology).

**LacZ immunohistochemistry.** Immunohistochemical staining was done according to standard procedures (22). We used a polyclonal rabbit anti-β-galactosidase IgG as primary antibody (ab616, dilution 1:1,500; Abcam) and a horseradish peroxidase-labeled polyclonal anti-rabbit IgG as secondary antibody (ab6221, dilution 1:500; Abcam). All slides were stained with diaminobenzidine (Dako). The intensity of

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**Fig. 1.** Study design for local jet injection (A) and analysis design for paraffin-embedded and shock-frozen tumor tissue (B). Resected tissue is divided for histopathologic evaluation or for cryosectioning of consecutive sections.
LacZ immunostaining in tumor cells as well as surrounding inflammatory cells was evaluated independently by two pathologists.

**X-Gal staining.** Cryosectioned tumor samples (7 μm) were fixed (2% formaldehyde, 10 min, 4°C), washed, and incubated with 3 mL X-Gal mix [1 mg/mL X-Gal, 1 mmol/L MgCl₂, 3 mmol/L K₃Fe(CN)₆, and 3 mmol/L K₄Fe(CN)₅, 24 h, 37°C] in quadriPERM plus chambers to develop blue staining. Samples were washed with PBS, covered with Faramount aqueous mounting medium (Dako), and evaluated with a light microscope.

**Capillary gel electrophoresis analysis of plasmid DNA topology.** Capillary gel electrophoresis of pCMVβ plasmid was done to analyze plasmid DNA topology shortly before and after jet injection. Plasmid DNA from the reservoir of the injector (before application) and the ejected plasmid DNA were analyzed. The plasmid pUK21 (PlasmidFactory) was used as a reference standard. Capillary gel electrophoresis analyses were done using a Beckman P/ACE MDQ instrument equipped with a LIF detector (488/520 nm) as described (24–26).

**Results**

**Patient characteristics.** Seventeen patients were treated between September 2005 and December 2006. One lesion per patient was selected for jet injection. All injected tumors were surgically resected 2 to 6 days after application. Four weeks after jet injection (last protocol consultation), all patients were alive and no patient presented any signs or symptoms related to the jet injection. The patients’ demographic and treatment variables are listed in Table 1.

**Safety.** Jet injection of LacZ plasmid DNA was well tolerated by 16 patients. One patient (patient 11) experienced acute pain during jet injection, resulting in a reduced dose application (discontinuation after three jet injections). No adverse events due to the jet injection were observed. Signs of small bleedings and jet penetration at the injection site disappeared within 48 h after jet injection (Fig. 2C). No significant changes of vital signs (heart rate, blood pressure, body temperature, and breathing rate) or of clinical laboratory variables were observed after jet injection.

**Plasmid DNA load in jet-injected tumors.** Plasmid DNA was present in all tumors with individual variations in the amounts detected. These variations were independent of tumor size or entity (Table 2; Fig. 3A).

Series of consecutive fractions of each cryosectioned tumor (see Fig. 1B) were analyzed to evaluate intratumoral distribution and load of jet-injected naked DNA. Detailed quantitative analyses of plasmid DNA load are shown for a representative patient of each tumor entity (patient 1, breast cancer; patient 2, melanoma) in Fig. 3B. Throughout the entire tumor tissues, presence of plasmid DNA was shown. Regarding the DNA level, great variations were detected in the consecutive tumor fractions. Areas with very high plasmid DNA load (21 ng to 132 pg/250 ng tissue DNA) were adjacent to areas with lower plasmid DNA load (0.7 to 0.2 pg/250 ng tissue DNA). Such inhomogeneous plasmid distribution was observed in tumors.
of all patients and is the result of the physical properties of the plasmid-dispersing jet. The plasmid DNA load is visualized in the corresponding agarose gel analyses, which validate the presence of the LacZ-specific amplicons in all tumor sections (Fig. 3B).

**LacZ-mRNA expression.** LacZ expression was detected and quantified in all 17 tumors, indicating the efficacy of jet-injection gene transfer (Fig. 3A). The LacZ-specific mRNA expression was detected throughout the entire tumors, as depicted for representative patients 1 and 2, and was verified by agarose gel electrophoresis (Fig. 3B). Comparison of plasmid DNA load in the tumor and the corresponding mRNA expression revealed a close correlation. This was supported by the data shown in Table 2, where highest peaks of plasmid DNA load result in highest LacZ-mRNA expression.

**LacZ-protein in tumor specimens.** LacZ-protein expression and distribution was found in jet-injected tumors of all patients by Western blot analysis, immunohistochemistry, and X-Gal staining (Table 2). The LacZ-protein expression for the tumor fractions (patients 1 and 2) with high LacZ-mRNA expression (shown in Fig. 3B) was confirmed by Western blot analysis. The expression of LacZ-mRNA corresponds to strong bands of LacZ-protein in the respective Western blots as shown for the first four fractions (see Fig. 3B). Insight into intratumoral distribution of LacZ-protein expression was

### Table 1. Characteristics of patients and treatment variables

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Karnofsky performance score (%)</th>
<th>Tumor entity</th>
<th>UICC tumor stage</th>
<th>Location of treated lesion</th>
<th>Time jet injection to resection (d)</th>
<th>Follow-up (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>82</td>
<td>F</td>
<td>100</td>
<td>Breast</td>
<td>IV</td>
<td>Thorax, L</td>
<td>3</td>
<td>18.4</td>
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<tr>
<td>2</td>
<td>67</td>
<td>F</td>
<td>90</td>
<td>Melanoma</td>
<td>IV</td>
<td>Thigh, L</td>
<td>3</td>
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</tr>
<tr>
<td>3</td>
<td>46</td>
<td>M</td>
<td>100</td>
<td>Melanoma</td>
<td>IIc</td>
<td>Inguinal/thigh, L</td>
<td>3</td>
<td>14.7</td>
</tr>
<tr>
<td>4</td>
<td>49</td>
<td>F</td>
<td>90</td>
<td>Breast</td>
<td>IV</td>
<td>Thorax, R</td>
<td>5</td>
<td>14.4</td>
</tr>
<tr>
<td>5</td>
<td>61</td>
<td>M</td>
<td>100</td>
<td>Melanoma</td>
<td>IIIc</td>
<td>Thigh, L</td>
<td>4</td>
<td>14.4</td>
</tr>
<tr>
<td>6</td>
<td>53</td>
<td>M</td>
<td>90</td>
<td>Melanoma</td>
<td>IIIc</td>
<td>Thigh, R</td>
<td>6</td>
<td>13.9</td>
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<td>7</td>
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<td>M</td>
<td>100</td>
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<td>IIb</td>
<td>Inguinal/thigh, L</td>
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<td>85</td>
<td>F</td>
<td>90</td>
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<td>Lower leg, R</td>
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<td>M</td>
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<td>8.9</td>
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<td>Melanoma</td>
<td>IIIc</td>
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<td>F</td>
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<td>F</td>
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<td>2</td>
<td>4.4</td>
</tr>
<tr>
<td>17</td>
<td>82</td>
<td>F</td>
<td>100</td>
<td>Melanoma</td>
<td>IIb</td>
<td>Thigh, R</td>
<td>3</td>
<td>4.2</td>
</tr>
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</table>

*The size of the tumor samples is given in length × height × depth.

### Table 2. Maximum plasmid DNA load and maximum relative LacZ mRNA, and protein expression in jet-injected tumors

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Maximum plasmid DNA load (pg/250 ng tissue DNA)</th>
<th>Relative LacZ-mRNA expression (in % calibrator)</th>
<th>LacZ-protein expression (immunohistochemistry)</th>
<th>Functional LacZ-protein expression (X-Gal staining)</th>
<th>Sample size (mm)*</th>
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<tr>
<td>1</td>
<td>132.8</td>
<td>7.8</td>
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<td>+</td>
<td>46 × 5 × 5</td>
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<tr>
<td>2</td>
<td>21066.1</td>
<td>863</td>
<td>+</td>
<td>+</td>
<td>18 × 5 × 9</td>
</tr>
<tr>
<td>3</td>
<td>&lt;0.001</td>
<td>0.6</td>
<td>+</td>
<td>+</td>
<td>54 × 5 × 25</td>
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<tr>
<td>4</td>
<td>8.9</td>
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<td>+</td>
<td>8 × 6 × 5</td>
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<tr>
<td>5</td>
<td>1676.9</td>
<td>723.5</td>
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<td>+</td>
<td>30 × 10 × 3</td>
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<tr>
<td>6</td>
<td>0.12</td>
<td>0.3</td>
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<td>+</td>
<td>20 × 10 × 10</td>
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<tr>
<td>7</td>
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<tr>
<td>8</td>
<td>0.17</td>
<td>&lt;0.1</td>
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<td>+</td>
<td>36 × 15 × 15</td>
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<td>9</td>
<td>135.2</td>
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<td>+</td>
<td>+</td>
<td>30 × 10 × 10</td>
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Abbreviations: M, male; F, female; L, left; R, right.
Fig. 3. A, plasmid DNA load and LacZ-mRNA expression in all jet-injected tumors (n = 17). Plasmid DNA presence is shown by amplicons of LacZ-specific PCR (top) and LacZ-mRNA expression by amplicons of RT-PCR (bottom). The agarose gel electrophoreses show amplicons of tumor fractions with highest values determined by the respective qPCR or qRT-PCR in Table 2. B, molecular biological analyses of two representative patients. Plasmid DNA load and LacZ expression in series of tumor fractions of skin metastases from breast cancer (patient 1) and melanoma (patient 2). Plasmid DNA load determined by qPCR and by agarose gel electrophoresis. Columns marked with asterisk are <40 pg plasmid DNA. LacZ-mRNA expression in these tumor fractions determined by qRT-PCR and by agarose gel electrophoresis. Columns marked with asterisk have relative LacZ expression levels ≤1% of the calibrator. Western blots of corresponding tumor fractions (as shown for fractions 1-4 for LacZ-mRNA expression) for LacZ-protein detection. C, localization of LacZ-protein expression by immunohistochemistry (magnification, ×200; inset, ×400) and by X-Gal staining (magnification, ×100). D, time course of plasmid DNA clearance in blood samples collected at indicated time points before and after jet injection determined in duplicates by qPCR.

M, DNA marker, 1 kb ladder (Fermentas); p.c., positive control; n.c., negative control.
provided by immunohistochemistry and X-Gal staining (Fig. 3C). Although jet injection creates circumscribed transfected tumor areas, they were found throughout the entire tissues by immunohistochemistry and X-Gal staining, showing deep penetration of jet-injection gene transfer. Despite the scattered distribution of LacZ expression, a high percentage of those areas that received the gene construct efficiently express the transgene.

Performing immunohistochemistry, LacZ expression was predominantly localized in the tumor cells (mainly in the cytoplasm and nuclear envelope), whereas some adjacent stromal and endothelial cells stained weakly (Fig. 3C). X-Gal staining showed inhomogeneous spotted distribution of the blue-stained areas at varying staining intensities, supporting the data determined by quantitative real-time PCR (qPCR) and qRT-PCR (Fig. 3C). Furthermore, the X-Gal staining was indicative of intratumoral expression of functional LacZ-protein.

**Plasmid DNA clearance from blood.** Beside plasmid DNA distribution in the tumor, its appearance and clearance in the blood is of clinical importance for the safety of this technology. Fourteen patients showed very similar kinetics of plasmid appearance 30 min after jet injection. This was followed by the very rapid decline within few hours. The time course of plasmid clearance is depicted for the two representative patients (Fig. 3D). The systemic plasmid DNA levels were very low at only femtogram amounts and showed no correlation between systemic peak plasmid DNA levels and DNA load in the tumors (Table 3). The individual time of complete clearance was also independent of the plasmid DNA peak levels in the blood. Blood analysis 4 weeks after jet injection did not show systemic plasmid DNA in any patient, which proved complete clearance from the blood compartment.

**Plasmid DNA topology.** Plasmid topology analysis by capillary gel electrophoresis revealed that preservation of GMP quality of plasmid DNA shortly before application is reflected by the high proportion of 97.1% covalently closed circular form of the plasmid (Supplementary Fig. S1A). Jet injection was accompanied with losses of closed circular plasmid (to a mean of 81.9%) and increase of the open circle form (from mean 1.8% to 16.2%) as an effect of shear forces during jet injection-mediated ejection (Supplementary Fig. S1B).

**Discussion**

The major challenge in clinical gene therapy is the assurance of safety and transfer efficiency. In this phase I trial, we proved for the first time the safety and efficacy of nonviral intratumoral jet injection of naked LacZ-expressing plasmid DNA. The application was well tolerated by all patients and histopathology showed no jet injection-related tissue damage. Expression and function of the LacZ reporter gene was shown in all treated tumors.

We showed that single application of only small amounts (50 μg) of plasmid DNA leads to efficient transgene expression. In comparison, other nonviral gene transfer studies of needle injection or needleless application of naked DNA required significantly more plasmid DNA (>1 mg) to achieve efficient gene expression (27, 28). The effectiveness of gene transfer observed with jet injection is attributed to the very high pressure applied to the liquid jet for a very short time to penetrate the tissue (29). It is proposed that higher liquid pressure applied to transfected tissues is decisive for more efficient gene transfer (18). Similar correlation of intercellular pressure and transfer efficiency was also shown for the hydrodynamic gene delivery (14, 30).

The resection of the jet-injected lesions provided the chance to obtain valuable quantitative and qualitative data on plasmid DNA loads and to correlate this to LacZ mRNA and protein expression. Analyses of serial tumor fractions revealed broad LacZ expression throughout the tumor at varying expression levels. This is in agreement with our previous preclinical data (18, 22). We showed the correlation between the intratumoral level of plasmid DNA load and the corresponding transgene

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Maximum plasmid DNA load (fg/250 ng blood DNA)*</th>
<th>Time of maximum plasmid DNA load (hours after jet injection)</th>
<th>Time of complete plasmid DNA clearance (hours after jet injection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>169.2</td>
<td>0.5</td>
<td>&gt;72</td>
</tr>
<tr>
<td>2</td>
<td>394.4</td>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>253</td>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>119.7</td>
<td>0.5</td>
<td>72</td>
</tr>
<tr>
<td>5</td>
<td>100.1</td>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
<td>96.6</td>
<td>0.5</td>
<td>72</td>
</tr>
<tr>
<td>7</td>
<td>27.3</td>
<td>3</td>
<td>72</td>
</tr>
<tr>
<td>8</td>
<td>162.8</td>
<td>3</td>
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<tr>
<td>9</td>
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<td>0.5</td>
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</tr>
<tr>
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<td>3</td>
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<tr>
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<tr>
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<tr>
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<td>87.1</td>
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<tr>
<td>15</td>
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<td>24</td>
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<tr>
<td>16</td>
<td>8485</td>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td>17</td>
<td>251.3</td>
<td>0.5</td>
<td>6</td>
</tr>
</tbody>
</table>

*DNA values are corrected by the recovery factor (see Patients and Methods).

Complete clearance is defined as time point when no plasmid DNA is detectable by real-time PCR in the blood sample collected at a defined time point after jet injection.
mRNA and protein expression. Immunohistochemistry demonstrated expression of LacZ-protein mainly within tumor cells and X-Gal staining proved functionality of LacZ. The inhomogeneous distribution in the tumor detected by immunohistochemistry and X-Gal staining is attributed to the physical properties of the applied high-speed (>300 m/s) fluid jet as shown in earlier animal experiments (18, 31). Furthermore, structure and density of the targeted tissue influence jet dispersion resulting in differences of plasmid DNA load and LacZ expression level (21, 29, 32, 33).

Local gene transfer by either needle injection or injection-catheter placements achieved only restricted spatial nonviral or viral vector distribution, which is limiting transgene expression to small areas in vicinity to the application site (34–37). Our approach with five jet injections from different angles results in greater transfection areas with deeper intratumoral penetrations (>1 cm) and improved DNA dispersion. Although scattered in distribution, jet-injection gene transfer affects greater areas of the target tissue. We observed that a high percentage of those areas that received the gene construct efficiently express the transgene. Similar distribution pattern of transgene expression was seen in our preclinical studies if other transgenes (green fluorescent protein, cytosome deaminase, and tumor necrosis factor-α) were jet injected (18, 22, 23).

Blood clearance of the jet-injected DNA was an important criterion of the study. As we have shown, the clearance follows very strict and reproducible kinetics. The observed plasmid DNA clearance peaked 30 min after jet injection followed by a rapid drop indicating the short-term, blood perfusion-mediated biodistribution of plasmid DNA followed by rapid elimination. Similar observations were made in other nonviral but also viral gene transfer studies (38, 39). Results from our own preclinical animal studies showed that circulating plasmid DNA does not lead to transgene expression in distant organs (21). The blood perfusion-mediated systemic circulation at very low DNA level was not sufficient to generate transgene expression at nontarget distant sites (40).

In vivo transduction efficiency is one major barrier in cancer gene therapy and the key factor to establish clinical treatments (41). Preclinical studies and clinical trials showed the current problems of systemic application of viral or nonviral gene therapy (3). Thus, local gene transfer has become more attractive for immunostimulatory and direct tumor cell-killing strategies by applying vectors intratumorally or in close vicinity to the tumor (42). Still, there are limitations of the local approach, mainly the accessibility of lesions, which restricts the method to isolated superficial tumors. However, the concept could be a promising option especially in a multimodality therapy setting (e.g., lesions outside of the perfused area in limb perfusion of metastatic melanoma). This study will be the basis for a next step to determine the antitumor efficacy of jet injection-based gene transfer of a therapeutic gene in a multimodality approach for improved tumor treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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