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Gene transfer into solid tumours—is a special application device beneficial?

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Abstract

The replacement of inactivated tumour suppressor genes is a promising approach in cancer therapy. The aim of this study was to evaluate the influence of technical determinants on the efficiency of adenoviral-mediated gene transfer into solid tumours. Therefore, we compared the efficacy of two different injection needle types, a conventional needle and a modified needle characterised by perforations at the side of the shaft *in vivo*. The total amount of adenoviral vector DNA and the activity of the transferred reporter gene were quantitatively analysed by polymerase chain reaction (PCR) specific for the E4 region of the Ad vector genome and the β-galactosidase assay, respectively. The levels of adenoviral DNA, as well as the total β-galactosidase activity, varied widely, but were not significantly different for the two groups. These results suggest, that the efficiency of intratumoral gene transfer cannot be improved by the design of the application device.

Keywords: Gene therapy; Solid tumours; Intratumoral vector application

1. Introduction

Lung cancer is a prominent cause of cancer death in the Western world [1]. Despite aggressive multimodal therapeutic concepts including bronchoscopic interventions in advanced tumour stages, no significant improvement could be achieved concerning the poor prognosis of non-small cell lung cancer (NSCLC) [2,3]. One of the promising molecular strategies for the treatment of solid tumours, especially for NSCLC is the replacement of inactivated or deleted tumour suppressor genes [4–6]. In clinical trials, gene transfer into endobronchial tumour lesions was performed by intratumoral application of retroviral and adenoviral vectors using fibrebronchoscopes [7–10]. Here, successful gene transfer was proved in post-treatment biopsies using polymerase chain reaction (PCR), immunohistochemistry or enzymatic assays to evaluate the expression of the reporter gene *β-galactosidase* or the therapeutic gene *TP53* [8,9,11]. However, a considerable variability in the expression of the transgene has been described [8,11]. The variability of gene transfer is due to the influence of different factors. Since biopsies were taken from the margin of the tumour they may not be representative for the transduction of the whole tumour [8,12]. The gene transfer efficiency is also influenced by the different infectibility of the tumour cells, which is well documented for NSCLC cell lines [13,14]. Another limitation for the adenovirus-mediated gene transfer are host defence mechanisms arrayed against the adenoviral vector [15–17]. Furthermore, mechanical aspects of vector application and vector distribution in solid tumours may be of importance for the efficiency of the transduction [12,18]. Consequently, an application mode leading to a widespread and homogeneous distribution of the injected vector solution throughout the tumour nodule would improve gene transfer and induce more extensive bystander effects [19,21].

Until now, little is known about the role of mechanical determinants and application devices for the distribution of adenoviral vectors in tumour tissue. Preliminary experiments with different viscosities of the vector solution

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and with the performance of single or multiple needle passes did not have a relevant influence on the gene transfer efficiency. We therefore investigated in this study the influence of modified application devices.

2. Materials and methods

2.1. Application device

A conventional injection needle (23-gauge, 0.6×18 mm, Braun, Germany) was modified by laser beamed perforations at the side of the shaft (GIP Medizin Technik GmbH, Grassau, Fig. 1a–e). The needle used for the *in vivo* studies was characterised by perforations with a diameter of 0.15 mm at 5 mm distance from the closed tip.

2.2. Cells and vectors

The human NSCLC cell line H460 was purchased from the American Type Culture Collection (ATCC) (Cat. No. HTB-177). Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% fetal calf serum (FCS) and 2 mM-glutamine at 37 °C, 5% CO_2 in a humidified atmosphere.

The recombinant adenoviral vector coding for the *Escherichia coli β-galactosidase* gene (AdCMV.*β-gal*) was a gift from R. Crystal (Cornell University Medical College, New York, USA). The vector was propagated on 293 cells and purified by caesium chloride density centrifugation, titred by plaque assay and stored at −80 °C [21,22]. The amount of active virus particles is expressed as plaque forming units (pfu).

2.3. Animal model

For the *in vivo* experiment, 5×10^6 cells resuspended in 35 μl RPMI 1640 were inoculated subcutaneously (s.c.) into the flank of C57Bl6/Bom-nu/nu mice. After 13 days, tumours with diameters of approximately 1 cm were transduced with 1×10^8 pfu of AdCMV.*β-Gal* diluted in 200 μl phosphate-buffered saline [23]. All injections were done transcutaneously with the same constant low pressure over 1 min. After 24 h, the tumours were explanted, measured and immediately frozen in liquid nitrogen for further analysis. The tumour volume was calculated from two tumour dimensions following the formula for the volume of a prolate ellipsoid $L \times W^2/2$ (where L designates the longer axis).

2.4. Tissue preparation

The frozen tumours were weighed and triturated on ice. Homogenised tissue was incubated in lysis buffer (100 mM potassium phosphate pH 7.8, 0.2% Triton X-100, 1 mM DDT, 0.2 mM phenylmethyl sulphonyl fluoride (PMSF) and 5 μg/ml leupeptin) on ice for 90

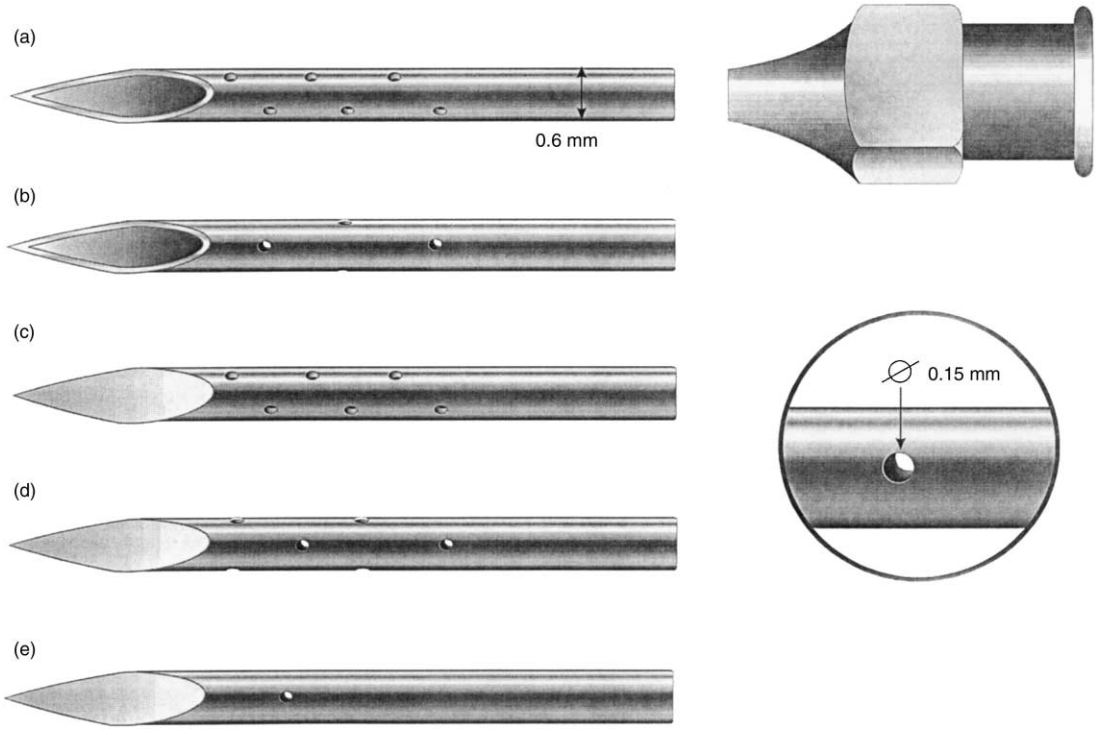

Fig. 1. Modified injection needles (23-gauge) were constructed to achieve a more homogeneous distribution of the injected solution beyond the needle track (a–e). The most effective needle type evaluated in an experimental setting was characterized by two laser-beamed small perforations perpendicular to the bevel. In order to increase the injection pressure, the tip was closed (e).

min. After inactivation of the endogenous β-galactosidase at 48 °C for 1 h, the solution was centrifuged (14 000 *g* at 4 °C for 5 min) and the supernatant was used for further analysis.

2.5. PCR analysis

We evaluated the total amount of the transduced virus DNA by semiquantitative PCR of the viral E4 region in comparison to the endogenous murine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. For this purpose, aliquots of the lysate were digested overnight with proteinase K and DNA was extracted using phenol-chloroform. Amplification was performed for *GAPDH* as the internal standard and the E4 region of the virus using the following primer sequences: *GAPDH* sense: 5-CAA GGC TGT GGG GAA GGT CA-3; *GAPDH* antisense: 5-ATG GGG GTC TGG GAT GGA AA-3; E4 sense: 5-GTA GAG TCA TAA TCG TGC ATC AGG-3; E4 antisense: 5-TTT ATA TGG TAC CGG GAG GAG GTG-3. All PCR procedures were performed in a final volume of 20 μl with NH_4Cl–buffer, 1.25 mM $MgCl_2$, 1.5 U Taq polymerase (all InViTek, Berlin, Germany), 200 μM deoxynucleotides (Stratagene, Heidelberg, Germany) and 10 pmol of each primer. After an initial denaturation at 94 °C for 5 min, cycling steps for *GAPDH* amplification were performed using the following programme: denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and elongation at 72 °C for 30 s. The same programme was used for E4 amplification except for the primer annealing which was performed at 58 °C.

For both genes to be amplified, a preliminary experiment was performed to verify the linearity of gene copy-number measurement. To this end, aliquots were taken after 20, 23, 26, 29, 32 and 35 cycles and analysed by densitometry. Optimal conditions were found to be 30 cycles for *GAPDH* and 32 cycles for E4. PCR products were resolved by electrophoresis in a 2% agarose gel containing ethidium bromide and the intensity of each band was measured by imaging analysis (Eagle Eye System with ONEDscan software, Stratagene). For generation of a standard curve, we used the DNA of H460 cells transduced with an adenoviral vector. The amount of viral DNA in relation to the amount of *GAPDH*-DNA is given as arbitrary units (aU).

2.6. Evaluation of transgene expression

The β-galactosidase activity was determined using a chemiluminescence assay (Galacto-Light™, Tropix, Inc. Bedford, MA, USA) according to the manufacturer's instructions with a MicroLumat LB 96 P (EG&G Berthold, Bad Wildbad, Germany). Protein concentrations were determined using a modified Lowry method (DC Protein Assay, Bio-Rad, Munich, Germany).

2.7. Analysis of the efficacy of transfection

In order to evaluate the relationship between the expression of the transgene and the amount of transduced Ad vector DNA, we determined the ratio of absolute β-galactosidase activity and the total amount of adenoviral DNA per tumour.

2.8. Statistical analyses

Data of both treatment groups failed the variance test and were evaluated by Mann–Whitney Rank Sum Test. The correlation between two parameters was analysed using Pearson's Correlation Coefficient. All analyses were performed with SigmaStat software (Statistical Package for the Social Sciences (SPSS) Software Service, Erkrath, Germany).

3. Results

Understanding of the mechanisms of injection and distribution of gene transfer vectors into solid tumours are fundamental in order to interpret gene therapy studies. Therefore, we investigated the effect of modification of the application device on the efficacy of gene expression after adenoviral-mediated gene transfer. In preliminary experiments, we compared the effects on the distribution of a coloured fluid injected into pectin gels using a conventional injection needle (23-gauge, 0.6×18 mm, Braun, Germany) or modified needles (same dimensions) with laser beamed perforations at the side of the shaft (Fig. 1a–e). Using the conventional needle, the injected fluid was concentrated near the tip only. Injections using needles with multiple perforations and open tips were less effective. Due to the decrease of the injection pressure, the fluid was not pressed into the gel, but mostly leaked out of the needle track. For a more widespread and homogenous distribution, a modified needle characterised by two perforations (diameter 0.15 mm) at 5 mm distance from the tip perpendicular to the bevel and a closed tip (Fig. 1e) proved to be the most effective.

In order to investigate differences in the distribution of the injected fluid *in vivo*, 200 μl of trypan blue was injected into four tumours using the conventional and the modified needle, respectively. Colouring of the tumour tissue was then compared. In contrast to the *in vitro* studies, there were no differences in the fluid distribution between both needles. With both instruments, the fluid was mainly placed in the necrotic and cavernous parts of the tumour.

For the *in vivo* evaluation of the gene transfer efficiency, both the conventional needle ($n=7$) and the modified needle ($n=8$) with the same diameter (23-gauge) were used. The vector solution was transcutaneously injected

into the tumours. Using the conventional needle, a ballooning of the subcutaneous tissue near the tip of the device was observed, despite the application of only 200 μl. Visibly, the tumours burst due to the hydrostatic pressure and injected fluid leaked from the tumour nodule into the subcutaneous space. When using the modified needle, less ballooning of the surrounding tissue was observed and only a small fraction of fluid leaked through the needle track.

3.1. Analysis of the Ad vector DNA per tumour

In order to see whether the macroscopically observed differences during the application of the vector solution resulted in differences in transfection we evaluated the total amount of the transduced viral DNA. In tumours treated by the conventional needle, the total amount of viral DNA varied between 22.7 aU and 71.6 aU per tumour, median 53.6 aU. In tumours treated by the modified needle, the median amount of viral DNA was 11.7 aU per tumour, with a minimum of 4.5 aU and a maximum of 32.2 aU (Fig. 2a). The conventionally treated tumours showed statistically significantly higher total amounts of viral DNA than tumours treated by the modified needle ($P > 0.05$ determined by Mann–Whitney rank sum test).

The tumour volume, calculated by measuring two diameters of the tumour nodule was closely correlated to the tumour mass determined by weighing. The total amount of adenoviral DNA did not show any correlation with either the tumour mass or the tumour volume, respectively (data not shown). In the conventionally-treated group, the amount of viral DNA per tumour mass varied between 18.0 and 113.3 aU/mg (median 5.6 aU/mg). In tumours treated with the modified needle, amounts of Ad vector DNA per tumour mass ranged between 3.7 and 86.7 aU/mg, median 31.0 aU/mg. There was no significant difference between the groups ($P = 0.08$, data not shown).

3.2. Analysis of transgene expression per tumour

Since the therapeutic effect depends on the transgene expression, we next determined whether there were differences in the protein expression of the reporter gene. The absolute β-galactosidase activity showed similar levels in the tumours treated by the conventional and the modified needle ($P = 0.35$, Mann–Whitney rank sum test). In tumours treated by the conventional needle, the median of absolute β-galactosidase activity was 2392.3 mU with values varying between 343.3 and 7304.9 mU. Using the modified needle, the absolute activity of the transduced β-galactosidase showed a smaller variance. The median was 1463.0 mU with values ranging from a minimum of 210.3 mU to a maximum of 2572.4 mU per tumour (Fig. 2b).

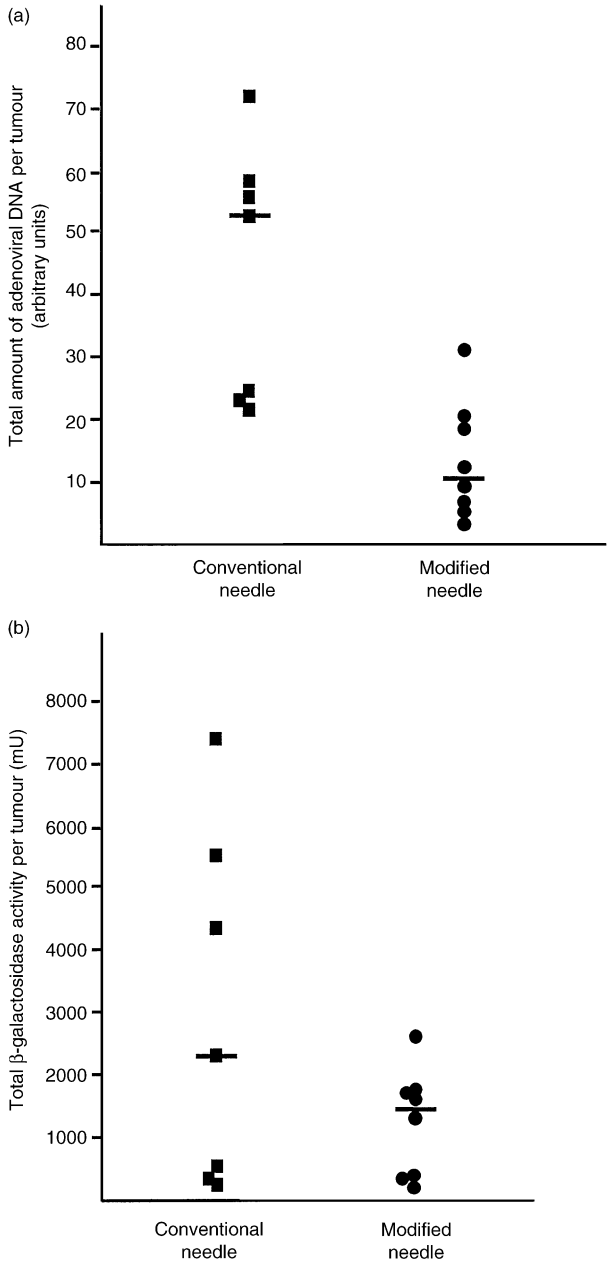

Fig. 2. (a) Total amount of Ad vector DNA per tumour. The total amount of adenoviral DNA per tumour was semiquantitatively evaluated by the polymerase chain reaction (PCR) 24 h after a single injection of AdCMV. β-Gal (10^8 plaque forming unit (pfu)). The transduced vector DNA was standardised to endogenous *GAPDH*-DNA and quantitated against a standard curve using DNA from H460 cells transduced with an adenoviral vector. Data are designated as arbitrary units (all). (b) Total amount of β-galactosidase activity per tumour. Quantitative analysis of total β-galactosidase activity per tumour 24 h after a single injection with AdCMV.β-Gal (10^8 plaque forming unit (pfu)). Enzyme activity was measured with a chemiluminescence assay and quantitated with the aid of a purified enzyme standard. Activity is given in units (mU).

In order to evaluate the relationship of transgene expression to the tumour mass, we also determined the relative β-galactosidase activity per tumour mass. In the conventionally-treated group, the median β-galactosidase

activity per tumour mass amounted to 1.9 mU/mg (minimum 0.4 mU/mg, maximum 11.9 mU/mg). In tumours treated by the modified needle, β-galactosidase activity per tumour mass ranged between 0.3 and 3.6 mU/mg with a median of 2.1 mU/mg. No significant differences could be confirmed between the groups (Fig. 3a). Statistical analysis did not confirm a correlation between β-galactosidase activity and the tumour mass (Pearson's correlation coefficient $r = 0.20$ for tumours treated with the modified needle, $r = 0.19$ for tumours treated with the conventional needle) (Fig. 3b and c) or the tumour volume (data not shown), independent of the application device used.

3.3. Analysis of the efficacy of transfection

Finally, the ratio of transgene expression and transduced Ad vector DNA was determined. In the conventionally-treated group, the median of absolute β-galactosidase activity per total amount of Ad vector DNA was 77.5 mU/aU. Data varied between 4.8 and 285.0 mU/aU. Tumours treated by the modified needle showed a median ratio of 82.0 mU/aU, with values ranging between 20.8 and 444.7 mU/aU. No significant differences were determined between the groups ($P = 0.39$, Mann–Whitney rank sum test) (Fig. 4).

4. Discussion

In principle, it is evident that a single vector injection is sufficient to transduce approximately fifty percent of cells in the centre of the tumour [24–26]. However, in clinical studies a considerable variance of the transgene expression in tumour biopsies was found among patients treated with equal doses of virus [8,27]. The fraction of infected tumour cells per biopsy ranged between 5 and 55% [8]. Until now, it could not be proved whether these differences reflect the real transduction rate or whether the transduced cells are focally distributed within the tumour. The efficiency of gene transfer into endobronchial tumours is one of the major concerns in gene therapy for NSCLC [12,18]. Moreover, the clinical and bronchoscopical situation, particularly in cases of advanced lung cancer located in the central tracheobronchial system, strongly demands effective and safe application devices [28,29]. Thus far, all investigators have used conventional needles (21-gauge) for local virus application [8,9,24,25].

In order to optimise gene transfer into endobronchial tumours, we constructed several types of injection needles. Since previous studies demonstrated that the distribution of transduced cells was consistent with the needle track [24,30], our intention focused on a more widespread and homogenous distribution of the injected fluid. Although this concept was effective in *in vitro* experiments, the

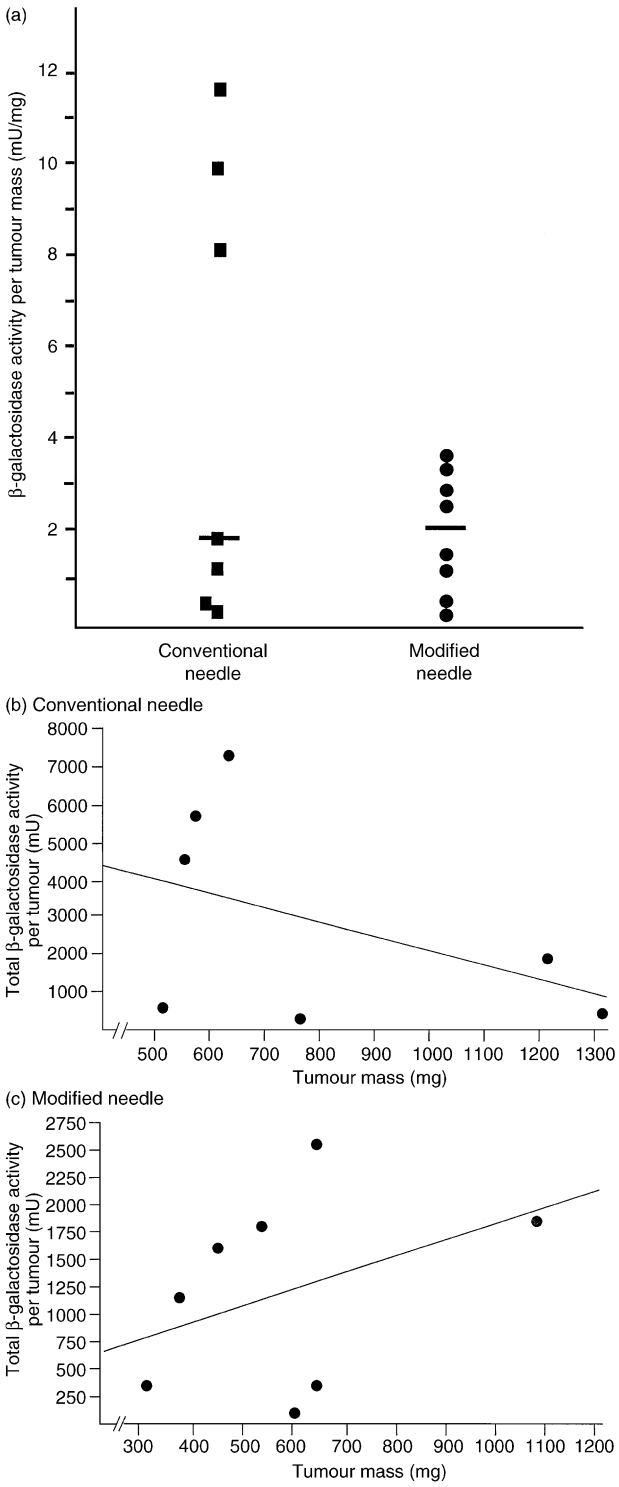

Fig. 3. (a) Relative β-galactosidase activity per tumour mass. Tumour mass was determined by weighing the explanted tumour and correlating this with β-galactosidase activity as determined in (b). Enzyme activity is given as mU/mg. (b) and (c) β-galactosidase activity per tumour in relation to the tumour mass. The total amount of β-galactosidase activity per tumour versus the tumour mass for the individual tumours were determined and the regression lines for the two groups treated with the conventional needle (b) and the modified needle (c) were calculated.

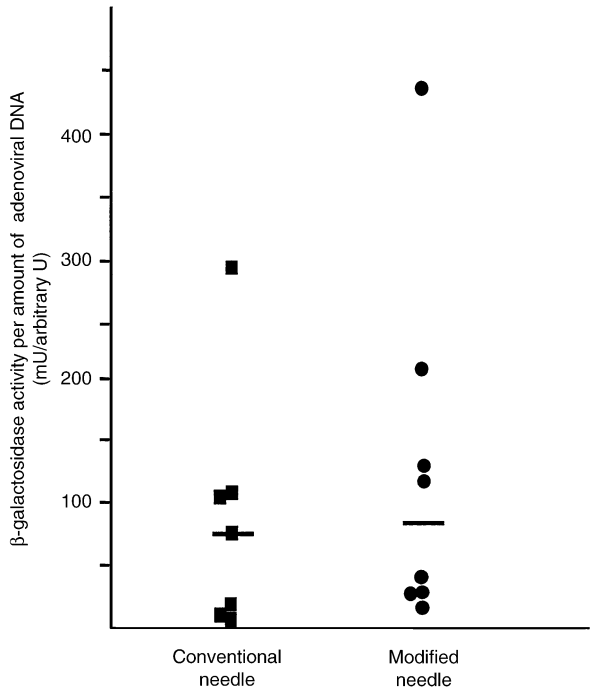

Fig. 4. Total β-galactosidase activity in relation to the total amount of Ad vector DNA. The total enzyme activity per tumour is correlated with the total amount of transduced Ad vector DNA present in the tumour. Data are given as mU/aU.

effect concerning transfer efficiency failed *in vivo*. The total amount of viral DNA and the β-galactosidase activity per tumour varied widely in both groups, particularly in the conventionally-treated group. In tumours treated with the modified needle, the data showed less variance, but did not reach the maximum values of the conventionally-treated group. However, these differences were not statistically significant.

Since transgene expression first of all correlates with the number of transfected cells, several reasons for the wide variance of β-galactosidase activity have to be taken into account [20,24]. Biological determinants of gene transfer efficiency such as clearance of the adenovirus by the innate immune system were similar for each tumour [15,17]. Physical reasons like leakage during the injection procedure may count as the main reasons for this variability.

For quantitative evaluation of the gene transfer efficiency, histochemical staining of the tumour sections for β-galactosidase activity has been used [24–26]. In our study, we did not observe a difference in the liquid distribution as measured by trypan blue injection (data not shown). Therefore, in this study the total amount of viral DNA, as well as the total β-galactosidase activity of the whole tumour, were determined for the evaluation of the gene transfer efficiency. Interestingly, a remarkable discordance between both of these parameters was found. Obviously, the level of transgene expression was independent of the amount of transfected vector DNA. Similar effects were shown by Tursz and Gahèry-Sègard and colleagues in clinical studies [8,27]. The reason for this phenomenon remains unclear. Evidently, the expression of the transgene has the greatest impact for evaluation of the transfer efficacy.

In contrast to clinical studies, in animal models genetically identical cells were transfected, thus differences in the infectibility of cells were minimised. As potential mechanical determinants for transfer efficiency tumour mass and tumour volume were included in the evaluation. Large tumours theoretically afford the uptake of a larger volume of the injected fluid leading to a higher transduction rate than smaller tumours. However, our results indicate that the transduction rate is independent of the tumour volume and the tumour mass. The wide variance of transfer efficiency even under standardised experimental conditions must be determined by the characteristics of the tumour tissue. Our observations during intralesional injections support this view that the tumour consistence is relevant for the effective application of the vector-carrying fluid. Tumours frequently burst and a small fraction of the injected fluid leaked out into the surrounding tissue. In cases of firm tumour consistence, the fluid leaked out of the needle track. This effect was independent of the device used. Obviously, the increasing hydrostatic pressure led to leakage at areas of low resistance. The loss of the injected fluid due to increasing hydrostatic pressure was also observed by Cusack and colleagues, who described an increased leakage after multiple needle passages [24].

Using a constant injection volume of 200 μl, we observed that the efficiency of gene transfer into solid tumours could not be significantly influenced by modifications of the application device. This result is in agreement with other technical investigations demonstrating that other application modes like injection by multiple needle passages or dividing the dose into equal fractions administered sequentially have also been shown not to improve gene transfer efficiency [24].

In summary, our study shows that technical modifications of the application device do not improve the efficiency of therapeutic gene transfer into solid tumours. For the transduction of solid tumours, the use of conventional injection needles with a small diameter (25–23-gauge) can be recommended.

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References

1. Parkin DM, Pisani P, Ferlay J. Global cancer statistics. *CA Cancer J Clin* 1999, **49**, 1411–1422.
2. Hoffmann PC, Mauer AM, Vokes EE. Lung cancer. *Lancet* 2000, **355**, 479–485.
3. Ernster VL, Mustacchi P, Osann KE. Epidemiology of lung cancer. In Murray JF, Nadel JA, eds. *Textbook of Respiratory Medicine*. Philadelphia, W.B. Saunders Company, 1994, 1504–1527.
4. Rom NW, Hay JG, Lee TC, Jiang Y, Tchou-Wong KM. Molecular and genetic aspects of lung cancer. *Am J Respir Crit Care Med* 2000, **161**, 1355–1367.
5. Bunn PA, Soriano A, Johnson G, *et al*. New therapeutic strategies for lung cancer. *Chest* 2000, **117**, 163S–168S.
6. Roth JA, Cristiano RJ. Gene therapy for cancer: what have we done and where are we going? *J Natl Cancer Inst* 1997, **98**, 21–39.
7. Roth JA, Nguyen D, Lawrence DD, *et al*. Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer. *Nat Med* 1996, **2**, 985–991.
8. Gahèry-Sègard H, Molnier-Frenkel V, Le Bolaire C, *et al*. Phase I Trial of recombinant adenovirus gene transfer in lung cancer. Longitudinal study of the immune responses to transgene and viral products. *J Clin Invest* 1997, **100**, 2218–2226.
9. Roth JA, Swisher SG, Merrit JA, *et al*. Gene therapy for non-small cell lung cancer: a preliminary report of a phase I trial of adenoviral p53 gene replacement. *Sem Oncol* 1998, **3**, 33–37.
10. Nemunaitis J, Swisher SG, Timmons TD, *et al*. Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. *J Clin Oncol* 2000, **91**, 609–622.
11. Swisher SG, Roth JA, Nemunaitis J, *et al*. Adenovirus-mediated p53 gene transfer in advanced non-small-cell lung cancer. *J Natl Cancer Inst* 1999, **91**, 763–771.
12. Roth JA. Gene replacement strategies for lung cancer. *Curr Opin Oncol* 1998, **10**, 127–132.
13. Batra RK, Olsen JC, Pickles RJ, *et al*. Transduction of non-small cell lung cancer cells by adenoviral and retroviral vectors. *Am J Respir Cell Mol Biol* 1998, **18**, 402–410.
14. Zhang WW, Fang X, Mazur W, *et al*. High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus. *Cancer Gene Ther* 1994, **1**, 5–13.
15. Yang Y, Nunes FA, Berencsi K, *et al*. Cellular immunity to viral antigenes limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci USA* 1994, **91**, 4407–4411.
16. Wolff G, Worgall S, Van Rooijen N, *et al*. Enhancement of in vivo adenovirus-mediated gene transfer and expression by prior depletion of tissue macrophages in the target organ. *J Virol* 1997, **71**, 624–629.
17. Worgall S, Wolff G, Falck-Pedersen E, *et al*. Innate immune mechanisms dominate elimination of adenovirus vectors following in vivo administration. *Hum Gene Ther* 1997, **8**, 37–44.
18. Frederiksen KS, Petri A, Abrahamsen N, *et al*. Gene therapy for lung cancer. *Lung Cancer* 1999, **23**, 191–207.
19. Wu JK, Cano WG, Meylaerts SAG, *et al*. Bystander tumoricidal effect in the treatment of experimental brain tumors. *Neurosurgery* 1997, **35**, 1094–1103.
20. Heise CC, Williams A, Olesch J, *et al*. Efficiency of a replication-competent adenoviraus '(ONYX-015) following intratumoral injection: intratumoral spread and distribution effects. *Cancer Gene Ther* 1999, **6**, 499–504.
21. Rosenfeld MA, Siegfried W, Yoshimura K. Adenovirus-mediated transfer of a recombinant alpha 1-antitrypsin gene to lung epithelium in vivo. *Science* 1991, **252**, 431–434.
22. Rosenfeld MA, Yoshimura K, Trapnell BC, *et al*. In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell* 1992, **68**, 143–155.
23. Zhang WW, Roth JA. Methods for cancer gene therapy using tumor suppressor genes. In Robbins PD, ed. *Methods in Molecular Medicine. Gene Therapy Protocols*. Totowa, NJ, Humana Inc., 1997, 47–59.
24. Cusack JC, Spitz FR, Nguyen D, *et al*. High levels of gene transduction in human lung tumors following intralesional injection of recombinant adenovirus. *Cancer Gene Ther* 1996, **3**, 245–249.
25. Hirschowitz EA, Ohwaha RP, Russi TJ, *et al*. In vivo adenovirus-mediated gene gene transfer of the Escherichia coli cytosine deaminase gene to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. *Hum Gene Ther* 1995, **50**, 437–447.
26. Brody SL, Jaffe HA, Han SK, *et al*. Direct in vivo gene transfer and expression in malignant cells using adenovirus vectors. *Hum Gene Ther* 1994, **5**, 437–447.
27. Tursz T, Le Cesne A, Baldeyrou P, *et al*. Phase I study of a recombinant adenovirus-mediated gene transfer in lung cancer patients. *J Natl Cancer Inst* 1996, **88**, 1857–1863.
28. Witt C, Ortner M, Ewert R, *et al*. Multiple fistulas and tracheobronchial stenoses require extensive stenting of central airways and esophagus in squamous cell carcinoma. *Endoscopy* 1996, **28**, 381–385.
29. Witt C, Dinges S, Schmidt B, *et al*. Temporary tracheobronchial stenting in malignant stenoses. *Eur J Cancer* 1997, **33**, 204–208.
30. O'Malley BW, Chen HS, Schwartz MR, *et al*. Adenovirus-mediated gene therapy for human head and neck squamous cell cancer in a nude mouse model. *Cancer Res* 1995, **55**, 1080–1085.