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In vivo induction of interferon gamma expression in grey horses with metastatic melanoma resulting from direct injection of plasmid DNA coding for equine interleukin 12

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Abstract

Whole blood pharmacokinetics of intratumourally injected naked plasmid DNA coding for equine Interleukin 12 (IL-12) was assessed as a means of in vivo gene transfer in the treatment of melanoma in grey horses. The expression of induced interferon gamma (IFN-γ) was evaluated in order to determine the pharmacodynamic properties of *in vivo* gene transduction. Seven grey horses bearing melanoma were injected intratumourally with 250 µg naked plasmid DNA coding for IL-12. Peripheral blood and biopsies from the injection site were taken at 13 time points until day 14 post injection (p.i.). Samples were analysed using quantitative real-time PCR. Plasmid DNA was quantified in blood samples and mRNA expression for IFN-γ in tissue samples. Plasmid DNA showed fast elimination kinetics with more than 99 % of the plasmid disappearing within 36 hours. IFN-γ expression increased quickly after IL-12 plasmid injection, but varied between individual horses. Intratumoural injection of plasmid DNA is a feasible method for inducing transgene expression in vivo. Biological activity of the transgene IL-12 was confirmed by measuring expression of IFN-γ.

Keywords: melanoma, horse, immune therapy, DNA transfection, real-time PCR

Direkte Injektion von Plasmid DNA kodierend für equines Interleukin 12 induziert *in vivo* eine Interferon gamma Expression bei Schimmeln mit Melanomen

Die Pharmakokinetik intratumoral injizierter nackter Plasmid DNA kodierend für equines Interleukin 12 (IL-12) wurde im Blut von Schimmeln mit Melanomen bestimmt um den *in vivo* Gentransfer durch diese Behandlung zu ermitteln. Zur Untersuchung der pharmakodynamischen Eigenschaften wurde die Expression von induziertem Interferon gamma (IFN-γ) quantifiziert. Sieben Schimmeln mit natürlich vorkommenden Melanomen wurden intratumoral 250 µg nackte Plasmid DNA kodierend für IL-12 injiziert. Vollblut und Biopsien aus der Injektionsstelle wurden zu 13 Zeitpunkten bis zum Tag 14 post injectionem entnommen. Die Proben wurden mittels quantitativer real-time PCR untersucht. Die Plasmid DNA wurde im Vollblut und die mRNA Expression des IFN-γ in den Gewebeproben quantifiziert. Plasmid DNA zeigte eine rasche Elimination aus dem Blut. Nach 36 Stunden waren weniger als 1 % der Plasmid DNA im Blut nachweisbar. Die IFN-γ-Expression stieg innerhalb kurzer Zeit nach der Plasmid-Injektion an, war aber individuell unterschiedlich. Die intratumorale Injektion von Plasmid DNA ist eine praktikable Methode zur in vivo Induktion transgener Expression. Die biologische Aktivität des transgenen IL-12 wurde durch den Nachweis der Induktion einer IFN-γ Expression belegt.

Schlüsselwörter: Melanom, Pferd, Immuntherapie, DNA Transfektion, real-time PCR

Introduction

Melanoma is one of the most common equine neoplasms with an incidence of 3.8 % to 15 % of all equine tumours (Sundberg et al., 1977; Valentine, 2006). Most promising therapeutic approaches try to support the patient's immune system in cancer suppression. Natural Killer (NK) cells and cytotoxic T-lymphocytes (CTLs) play a major role in the containment of tumour spreading (Beverley, 1995). One of the most promising cytokines in cancer therapy is interleukin 12 (IL-12) (Brunda et al., 1993; Colombo und Trinchieri, 2002). The gene therapeutic approach of IL-12 is well-tolerated and could provide a sustained expression and production of protein after local injection (Schultz et al., 2000; Heinzerling et al., 2001; Heinzerling et al., 2005). A number of investigations about pharmacokinetics of plasmid transfection have been published (Nishikawa et al., 2005b; Nishikawa et al., 2005a; Yew, 2005). Plasmid kinetics studies are of considerable importance in the development of therapeutically useful DNA vectors and application protocols (Nishikawa et al., 2005a). The aim of this study was to investigate the kinetics of intratumourally injected plasmid DNA coding for equine IL-12 to assess the possible duration of plasmid induced protein expression. Furthermore, the pharmacodynamic effect of the investigated DNA was analysed to evaluate its potential for clinical application.

Animals, Material and Methods

The study population consisted of 7 clinically healthy grey horses bearing melanoma. Six mares and one gelding, ranging from 8 to 21 years of age and 435 to 530 kg body weight, were used. The experimental procedure was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), approval no. 33.42502/05–15.05. At each study visit the horses were examined clinically. The injection site was checked for local reactions. Plasmid DNA (100 µl, 2.5 µg/µl) was injected with a pyrogen-free single use insulin syringe (B. Braun Melsungen AG, Melsungen, Germany) into the periphery of a suitable tumour (minimal size 1 cm^2). For injection, horses were sedated intravenously to individual needs (20–80 µg/kg body weight detomidin, Cepesedan®, CP-Pharma, Burgdorf, Germany). Four microbiopsies (approximately 50 µg) of the tumour at the site of injection were taken at each of 13 time points (directly prior to injection, at 10 and 30 minutes, 2, 4, 12, 24, 36 and 60 hours, 5, 7, 9 and 14 days post injection). Samples were used for total RNA isolation. After sampling, biopsies were shock-frozen immediately in liquid nitrogen at –197 °C and stored at –80 °C until further processing. Whole blood samples were drawn into EDTA-coated tubes at each time point in parallel to the biopsy samples. After allowing the blood to mix with

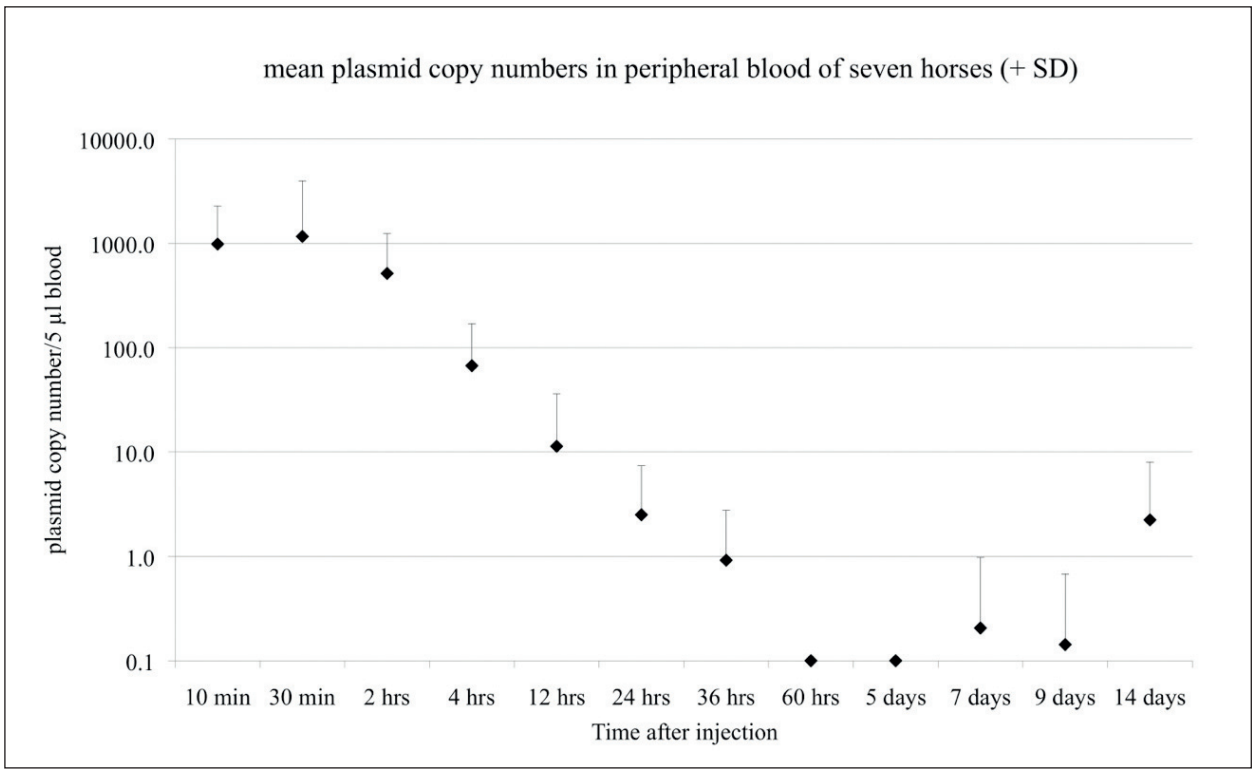

Figure 1: Mean plasmid copies recovered from peripheral blood following intratumoural plasmid injection. Error bars indicate the standard deviation. For better visualization they are only shown in own direction. Plasmid DNA detected in peripheral blood from 60 hours onwards was found in single horses only.

the anticoagulant, it was frozen at –197 °C and stored at –80 °C until further processing. The plasmid DNA construct pUSErIRESeIL12 for equine IL-12 was a kind gift from Dr L. Nicolson, University of Glasgow (via Intervet International, Boxmeer, The Netherlands) to the Equine Clinic of the University of Zurich. DNA was isolated from 200 µl EDTA-anticoagulated whole blood of all horses using the QiaAmp DNA Blood Mini kit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturer's instructions. Total RNA was isolated using the RNeasy Micro kit (Qiagen). cDNA was synthesized using DNAse treated RNA (12 µl) with the QuantiTect® Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Primers and probes for the detection of plasmid DNA and mRNA were designed to cover exon-exon junctions using the Primer Express™ software (Version 2, Applied Biosystems, Rotkreuz, Switzerland) to avoid amplification of genomic DNA.
Gene specific probes were labelled with reporter dye at the 5'-end. Real-time PCRs were performed using the ABI 7500 Fast Real-Time PCR system (Applied Biosystems) in the 9600 emulation mode. The fluorescence threshold value was calculated using the ABI 7500 Fast Real-Time PCR system software. All PCR reactions were performed in duplicate. Plasmid DNA from blood was quantified using the standard curve method as described elsewhere (Rutledge und Cote, 2003). To determine the relative expression of the gene of interest isolated from injected tumours, the comparative CT relative quantification method was used (Livak und Schmittgen, 2001). The obtained relative expression data are expressed as the fold change in gene expression normalized to the reference gene eGAPDH and relative to the untreated pre-injection sample.

Results

During the study period no adverse events were recorded. All 7 horses remained in good general condition. Results of the clinical examinations were within normal limits at each examination.

Plasmid recovered in peripheral blood

In the peripheral blood, plasmid DNA was detected as early as 10 minutes after intratumoural injection. It was measurable until 36 hours post injection. Thereafter, no plasmid was recovered except for three samples on days 7, 9, and 14 (ID nr. 3, 2, and 4, respectively). The peak concentration of $1.2x10^3 \pm 2.8x10^3$ plasmid copies/5 µl blood was reached 30 minutes p.i. (Fig 1). The following decline in plasmid DNA concentration showed a biphasic curve in individual horses.

Intratumoural expression of IFN-γ

IFN-γ expression was detectable in all the melanoma samples after in vivo transfection with the IL-12 plasmid DNA. However, an individually variable level of induction of IFN-γ expression was evident (Tab 1). Horse no. 2 showed a marked increase in IFN-γ expression after plasmid application with a more than 1000-fold increase in expression from the baseline 30 minutes after injection. The IFN-γ expression decreased with time, but remained elevated approximately 10-fold until the last sampling. In the other horses, the induction of IFN-γ expression was less marked and varied individually, but at 12 hours post injection a mean increase in IFN-γ mRNA expression of 21.8 ± 43.6 was observed. Horse no. 7 showed no induction of IFN-γ gene expression after injection (Tab 1).

Discussion

In the present study the tolerability and pharmacokinetics of intratumourally injected plasmid DNA coding for equine interleukin 12 (IL-12) and subsequent interferon-γ (IFN-γ) expression were investigated. Direct DNA injec-

Table 1: Interferon gamma expression in the individual study horses after single intratumoural injection of IL-12 plasmid DNA. Numbers show the relative fold increase of IFN-γ mRNA over baseline values. The highest value of the measured data during the first 24 hours is depicted. IFN-γ mRNA levels were measured using real-time two-step RT-PCR in tumour biopsies.

Horse no.	Max. expression during first 24 hrs	36 hrs	60 hrs	5 days	7 days	9 days	14 days
1	19.0	0.9	3.9	6.3	6.7	4.2	6.9
2	1618.0	12.8	42.7	47.7	38.5	2.6	42.5
3	109.5	2.6	3.0	0.4	6.7	1.4	6.4
4	5.8	4.6	15.8	3.8	0.6	2.7	0.3
5	15.5	3.2	0.5	6.1	0.9	15.3	17.5
6	6.6	1.4	1.3	48.7	0.3	0.9	0.7
7	1.1	0.1	1.0	0.8	0.1	0.2	0.9

tion proved to be a reliable and uncomplicated technique to achieve intratumoural gene transduction (Nomura et al., 1997). Because of its low immunogenicity, minimal toxicity and ease of use it has been widely applied in preclinical and clinical studies (Lin et al., 1990; Heinzerling et al., 2005). All horses used here were in good general condition during the entire study period and showed no serious adverse effects after injection of naked equine IL-12 plasmid DNA.

After injection, plasmid DNA was recovered from the peripheral blood. The venous appearance of plasmid DNA showed an approximately exponential decrease in concentration. After more than 36 hours the recovery of plasmid DNA from the blood was inconsistent. Three horses showed very low plasmid yields at 7, 9, and 14 days after injection, respectively. Two of the horses showed one peak, whereas the other five horses had a biphasic curve with two peaks. The first peak always appeared during the first 30 minutes after injection. This is probably due to plasmid DNA diffusion to blood vessels directly after intratumoural injection as stated elsewhere (Nishikawa und Hashida, 1999). It is known that a large part of injected plasmid DNA underlies rapid degradation in the tissue (Lechardeur et al., 1999). Only a small fraction of DNA is able to transfect host cells. Naked plasmid DNA can transfect various cell types, including tumour cells and inflammatory cells (Condon et al., 1996; Heinzerling et al., 2005). It is thus likely that in those horses in which a second peak of plasmid DNA was detected in the peripheral blood, this second peak might be due to the recovery of either phagocytising blood cells containing DNA fragments (detectable by our PCR primers), or that transfected inflammatory cells released into the blood were measured. From 60 hours (2.5 days) after plasmid injection onwards, hardly any plasmid DNA was recovered in the blood.

The efficiency of gene transfer is controlled by nuclear access, but also closely associated with the height and duration of transgene expression (Uherek und Wels, 2000; Nishikawa et al., 2005b). As the real-time PCR evaluation of transgene expression of plasmid coded IL-12 was not possible in this study due to suboptimal plasmid DNA digestion in the total RNA isolate, the true transfection efficiency cannot be estimated from the current data. To assess the production of biologically active transgene protein, the expression of its downstream mediator IFN-γ was analyzed. In our study population the increase in IFN-γ gene expression showed marked interindividual variability. The moderate increase in IFN-γ expression in most horses is comparable to earlier studies (Siddiqui et al., 2007). It suggests that direct intratumoural IL-12 plasmid injection leads to increased IFN-γ expression, probably due to the expression and production of biologically active IL-12 transgene and possibly also endogenous IL-12. One horse (horse no. 2) showed a more than 1000-fold increase in IFN-γ expression as a response to the IL-12 plasmid injection. It is worth to consider if this early and, in comparison to the remaining study horses, remarkably high rise in IFN-γ expression was due to an efficient transfection of host cells with IL-12 or due to an inflammatory reaction in the host tissue. In horse no. 7 no change in IFN-γ expression in relation to baseline levels was seen throughout the study period. This might be due to low transfection efficiencies in this horse or to a negative feedback mechanism of the secreted IL-12 subunits.

This investigation shows that the intratumoural injection of naked DNA coding for equine IL-12 in a watery solution can result in a marked upregulation in IFN-γ gene expression suggesting the ability of the pUSErIRESeIL12 plasmid DNA to transfect cells in vivo and express biologically active IL-12. The IFN-γ response varied within in the study population. Nevertheless, the horse with the highest IFN-γ response showed a sustained IFN-γ production during the study period. This shows that the injected dose can be sufficient to induce a desired immune response if circumstances are optimal.

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References

Beverley P.: Tumorimmunologie. In: Kurzes Lehrbuch der Immunologie. Hrsg. I.M. Roitt und J. Brostoff. Thieme Verlag, Stuttgart, 1995, 246–257.

Brunda M.J., Luistro L., Warrier R.R., Wright R.B., Hubbard B.R., Murphy M., Wolf S.F., Gately M.K.: Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J. Exp. Med. 1993, 178: 1223–1230.

Colombo M.P. und Trinchieri G.: Interleukin-12 in anti-tumor immunity and immunotherapy. Cytokine Growth Factor Rev. 2002, 13: 155–168.

Condon C., Watkins S.C., Celluzzi C.M., Thompson K. und Falo L.D., Jr.: DNA-based immunization by in vivo transfection of dendritic cells. Nat. Med. 1996, 2: 1122–1128.

Heinzerling L., Burg G., Dummer R., Maier T., Oberholzer P.A., Schultz J., Elzaouk L., Pavlovic J., Moelling K.: Intratumoral injection of DNA encoding human interleukin 12 into patients with metastatic melanoma: clinical efficacy. Hum. Gene Ther. 2005, 16: 35–48.

Heinzerling L.M., Feige K., Rieder S., Akens M.K., Dummer R., Stranzinger G. und Moelling K.: Tumor regression induced by

intratumoral injection of DNA coding for human interleukin 12 into melanoma metastases in gray horses. J. Mol. Med. 2001, 78: 692–702.

Lechardeur D., Sohn K.J., Haardt M., Joshi P.B., Monck M., Graham R.W., Beatty B., Squire J., O'Brodovich, H., Lukacs G.L.: Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. Gene Ther. 1999, 6: 482–497.

Lin H., Parmacek M.S., Morle G., Bolling S. und Leiden J.M.: Expression of recombinant genes in myocardium in vivo after direct injection of DNA. Circulation 1990, 82: 2217–2221.

Livak K.J. und Schmittgen T.D.: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001, 25: 402–408.

Nishikawa M. und Hashida M.: Pharmacokinetics of anticancer drugs, plasmid DNA, and their delivery systems in tissue-isolated perfused tumors. Adv. Drug. Deliv. Rev. 1999, 40: 19–37.

Nishikawa M., Takakura Y. und Hashida M.: Pharmacokinetics of plasmid DNA-based non-viral gene medicine. Adv. Genet. 2005a, 53: 47–68.

Nishikawa M., Takakura Y. und Hashida M.: Theoretical considerations involving the pharmacokinetics of plasmid DNA. Adv. Drug Deliv. Rev. 2005b, 57: 675–688.

Nomura T., Nakajima S., Kawabata K., Yamashita F., Takakura Y. und Hashida M.: Intratumoral pharmacokinetics and in vivo gene expression of naked plasmid DNA and its cationic liposome complexes after direct gene transfer. Cancer Res. 1997, 57: 2681–2686.

Rutledge R.G. und Cote C.: Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Res. 2003, 31: e93.

Schultz J., Heinzerling L., Pavlovic J. und Moelling K.: Induction of long-lasting cytokine effect by injection of IL-12 encoding plasmid DNA. Cancer Gene Ther. 2000, 7: 1557–1565.

Siddiqui F., Li C.Y., Larue S.M., Poulson J.M., Avery P.R., Pruitt A.F., Zhang X., Ullrich R.L., Thrall D.E., Dewhirst M.W., Hauck M.L.: A phase I trial of hyperthermia-induced interleukin-12 gene therapy in spontaneously arising feline soft tissue sarcomas. Mol. Cancer Ther. 2007, 6: 380–389.

Sundberg J.P., Burnstein T., Page E.H., Kirkham W.W. und Robinson F.R.: Neoplasms of Equidae. J. Am. Vet. Med. Assoc. 1977, 170: 150–152.

Uherek C. und Wels W.: DNA-carrier proteins for targeted gene delivery. Adv. Drug Deliv. Rev. 2000, 44: 153–166.

Valentine B.A.: Survey of equine cutaneous neoplasia in the Pacific Northwest. J. Vet. Diagn. Invest. 2006, 18: 123–126.

Yew N.S.: Controlling the kinetics of transgene expression by plasmid design. Adv. Drug Deliv. Rev. 2005, 57: 769–780.

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