J. Vet. Med. A **54,** 599–606 (2007)

ISSN 0931–184X
doi: 10.1111/j.1439-0442.2007.01002.x

Clinic of Small Animal Medicine, Ludwig Maximilian University Munich, Munich, Germany

Intra-tumoral Gene Delivery of feIL-2, feIFN-γ and feGM-CSF using Magnetofection as a Neoadjuvant Treatment Option for Feline Fibrosarcomas: A Phase-I Study

A. JAHNKE[1,6], J. HIRSCHBERGER[1], C. FISCHER[1], T. BRILL[2], R. KÖSTLIN[3], C. PLANK[2], H. KÜCHENHOFF[4], S. KRIEGER[4], K. KAMENICA[5] and U. SCHILLINGER[2]

Addresses of authors: [1]Clinic of Small Animal Medicine, Ludwig Maximilian University Munich (LMU), Veterinärstr. 13, 80539 Munich, Germany; [2]Institute of Experimental Oncology and Therapy Research, Technical University of Munich (TUM), Ismaningerstr. 22, 81675 Munich, Germany; [3]Department of Veterinary Surgery, LMU, Veterinärstr. 13, 80539 Munich, Germany; [4]Department of Statistics, LMU, Akademiestr. 1, 80799 Munich, Germany; [5]Institute of Veterinary Pathology, LMU, Veterinärstr. 13, 80539 Munich, Germany; [6]Corresponding author: Tel.: +49 179 2097038; fax: +49 89 21805913; Email: anika.jahnke@gmx.de

With 3 tables

Received for publication February 7, 2007

Summary

Despite aggressive pre- or postoperative treatment, feline fibrosarcomas have a high relapse rate. In this study, a new treatment option based on immune stimulation by intra-tumoral delivery of three feline cytokine genes was performed. The objective of this phase-I dose-escalation study was to determine a safe dose for further evaluation in a subsequent phase-II trial. Twenty-five client-owned cats with clinical diagnosis of fibrosarcoma – primary tumours as well as recurrences – entered the study. Four increasing doses of plasmids coding for feIL-2, feIFN-γ or feGM-CSF, respectively, were previously defined. In groups I, II, III and IV these doses were 15, 50, 150 and 450 μg per plasmid and a corresponding amount of magnetic nanoparticles. Two preoperative intra-tumoral injections of the magnetic DNA solution were followed by magnetofection. A group of four control cats received only surgical treatment. Side effects were registered and graded according to the VCOG-CTCAE scale and correlated to treatment. Statistical analyses included one-way ANOVA, *post hoc* and Kruskal–Wallis tests. ELISA tests detecting plasma feIFN-γ and plasma feGM-CSF were performed. One cat out of group IV (450 μg per plasmid) showed adverse events probably related to gene delivery. As these side effects were self-limiting and occurred only in one of eight cats in group IV, this dose was determined to be well tolerable. Altogether six cats developed local recurrences during a 1-year observation period. Four of these cats had been treated with dose IV. Regarding these observations, a subsequent phase-II trial including a representative amount of cats should be tested for the efficacy of dose IV as well as dose III.

Introduction

The existence of feline fibrosarcomas (FSA), especially feline vaccine- or injection-site associated sarcomas has been reported since the early 1990s (Hendrick and Goldschmidt, 1991). In the USA, the estimated incidence varies from 1/1000 to 2/10 000 vaccinated cats (Kass et al., 1993; Macy and Guillermo Couto, 2001). Likewise in Europe, FSA represents an everyday problem in veterinary practice. Despite the many clinical studies, which have been conducted in the search for a successful therapy, the healing prognosis for cats with FSA is still poor. Reported recurrence rates range from 41% to 69% (Quintin-Colonna et al., 1996; Cohen et al., 2001) even with adjuvant radiation- or chemotherapy. In human oncology, immunostimulatory gene therapy, especially by cytokine gene transfer, represents a promising treatment modality, mainly for solid tumours (Berzofsky et al., 2004). There are few publications about clinical trials dealing with gene therapy in companion animals. To the authors' knowledge, only two of these deal with feline fibrosarcomas: Quintin-Colonna et al. (1996) achieved promising results with local infiltration of xenogeneic cells secreting human interleukin-2 (huIL-2). The differences in recurrence rates of treated and control cats were statistically significant ($P < 0.007$). The recurrence rate of the group treated with surgery, radiation and additional immunotherapy (16 cats) was 31% versus 69% in the control group (16 cats) treated without additional gene therapy. Jourdier et al. (2003) treated cats with local injections of viral vectors expressing huIL-2 or feline (fe) IL-2 respectively (18 cats per group). The recurrence rates were 39% and 28% versus 61% in the control group, also consisting of 18 cats. At the Clinic of Small Animal Medicine (LMU Munich), a phase-I dose-escalation study using a combination of three feline cytokine genes for the adjuvant treatment of FSA has already been conducted. In this study a collagen sponge was implanted in the tumour bed immediately after surgery. This sponge had been loaded with plasmids coding for feIL-2, feline interferon-gamma (feIFN-γ) and feline granulocyte-macrophage colony-stimulating factor (feGM-CSF) (Kempf, 2005). These cytokines can enhance the immune responses by a variety of mechanisms.

Interleukin-2 initially described as 'T-cell growth factor' promotes the activation and maturation of T-helper cells (TH cells) and cytotoxic T cells (Cozzi et al., 1993). Furthermore, it stimulates growth and cytolytic activity of natural killer cells (NK cells) and macrophages. Together with IFN-γ, IL-2 induces the proliferation of B cells (Abbas et al., 2000).

IFN-γ is a potent inducer of major histocompatibility complex (MHC) class I molecules on tumour cells. By that, one of the most important tumour escape mechanisms can be overcome and the presentation of tumour antigens is permitted. Furthermore, the expression of MHC class II on dendritic cells (DC) is promoted so that the presentation of foreign antigens to effector cells is supported. In addition IFN-γ is a macrophage-activating cytokine and it promotes the development of TH1 cells, the cytolytic activity of NK cells and the isotype-switching of B cells (Abbas et al., 2000).

Granulocyte-macrophage colony-stimulating factor induces the proliferation and differentiation of cells committed to the granulocytes and macrophage lineage. By that, it promotes the maturation of monocytes and DC and hence increases phagocytotic and antigen-presenting activity (Abbas et al., 2000). Furthermore GM-CSF can up-regulate the expression of MHC- and costimulatory molecules (Armstrong and Hawkins, 2001).

All of these characteristics play important roles in antitumour immunity. The three described cytokines complement one another regarding their potential to activate an antitumour response as they activate cellular as well as humoral immune responses.

Additive immunostimulatory effects of the combination of IL-2 and IFN-γ as well as GM-CSF and IFN-γ have already been reported in murine tumours and it has been established that the therapeutic potential can be increased by combining certain cytokines in contrast to using a single cytokine (Rosenthal et al., 1994; Clary et al., 1996; Yoon et al., 1998). In this dose-escalation study, gene delivery of feIL-2, feIFN-γ and feGM-CSF was performed in feline FSA with the aim of establishing a safe dose for further exploration in a phase-II trial. The strategy of inducing the expression of immunostimulatory cytokines within tumour cells is called '*in situ* vaccination'.

Materials and Methods

Patient selection

Twenty-five client-owned cats with a clinical diagnosis of primary or recurrent fibrosarcoma were admitted to the study. A localization of the tumour at the trunk, the possibility of surgical removal in one session without necessary amputation, as well as a signed informed consent of the owner were the inclusion criteria. Exclusion criteria included detectable metastasis, medical history of other malignancy, present pregnancy, treatment with corticosteroids during the previous 6 weeks as well as prior radio-, chemo- or gene therapy. Furthermore, other severe underlying illnesses which lower life expectancy to <1 year had to be ruled out by anamnesis, clinical examination and blood tests. Complete blood counts (CBC), serum biochemical analyses including T4, FIV/FeLV and electrolytes were performed in each case on the day of first presentation.

Before enrolment all cats were staged by clinical examination, thoracic radiographs (three views) and abdominal ultrasonography. Tumours were measured with sliding calliper in three dimensions and staged according to Hirschberger and Kessler (2001). Their volume was calculated according to Chou et al. (1997). If possible, vaccination and previous injection histories were documented.

Assignment to treatment groups

This phase-I dose-escalation study was designed as a prospective study with four previously defined increasing doses of the mentioned cytokine gene combination. The choice of the initial dose (15 μg of each of the three plasmids or 45 μg total plasmid) was based on the total plasmid dose established by Dow et al. (1998) in dogs with oral melanoma and is 1/10 of the dose for small tumours, which was 400 μg. Doses were escalated approximately by factor 3 (15, 50, 150, 450 μg of each plasmid). Cats were entered serially upon dose escalation. At the beginning of the study it was determined that four cats had to be assigned to each of the four treatment groups named groups I, II, III and IV. There are no official regulations for phase-I trials regarding group sizes – neither for human nor for veterinary medicine. Small group sizes ranging from three to 10 probands are common. As signs of treatment related toxicity appeared in one cat of the highest dose group (group IV), it was decided to enlarge this treatment group by 100% (four more cats) so that a more representative group size was created and toxicity could be better evaluated. Neoadjuvant treatment was performed during a 2-week period before surgery as mentioned below.

For ethical reasons, it was refrained from administering empty plasmids to a control group and thus delaying surgery by 2 weeks. It was decided that four control cats should be treated with sole surgery. As a consequence these four cats were enrolled in a different manner forming a branch of study without pre-treatment so that surgery could be performed the day after first presentation. This control group can hence simply serve as a comparison for the postoperative period.

Owners as well as surgeons were blinded regarding the cats' affiliation to treatment and control group.

Plasmid solution and magnetofection

A non-viral gene transfer technology combining plasmids as gene vectors and the magnetofection method was used for neoadjuvant gene delivery. The plasmids p55pCMV_ivs_feIL-2, p55pCMV_ivs_feIFN-γ and p55pCMV_ivs_feGM-CSF were used under the control of the CMV (human cytomegalovirus) promoter which is often used as a standard promoter. These plasmids coding for feIL-2, feIFN-γ and feGM-CSF, respectively, were obtained from Plasmid Factory GmbH & CoKG (Bielefeld, Germany) in a concentration of 5.0 mg/ml in water each. Iron oxide magnetic nanoparticles coated with the polycation polyethylenimine were obtained from Chemicell GmbH (Berlin, Germany) in an aqueous suspension (60 mg nanomaterial per ml). Due to electrostatic interactions, these nanoparticles bind the negatively charged plasmid DNA when collectively brought into solution (Scherer et al., 2002). Equal amounts of the three different plasmids were combined and diluted to saline solution resulting in a volume of 250 μl. Amounts of magnetic nanoparticles equalling the total plasmid weight were diluted to 250 μl with water for injections. These two solutions were mixed in a 1:1 ratio using a micropipette so that in the end there was a total injection volume of 500 μl independent of the plasmid dose. After intra-tumoral (i.t.) injection of this magnetic DNA formulation, a neodymium-iron-boron magnet (NeoDeltaMagnet NE2010; IBS Magnet, Berlin, Germany) was attached to the tumour for 60 min. The

method of using magnetic force to target and enhance gene delivery is called magnetofection (Plank et al., 2003).

Treatment protocol

During the neoadjuvant treatment period, cats of the treatment groups received two i.t. injections of the magnetic DNA formulation: one on day −14 and one on day −7 before surgery. The doses of each plasmid were 15, 50, 150 and 450 μg in groups I, II, III and IV. Further dose escalation within the described study setting was not possible because of precipitation of the iron particles. Otherwise an increase of the injection volume to 1000 μl would have been necessary, which was undesired and not practicable in smaller tumours.

After each of the i.t. injections a neodymium–iron–boron magnet was attached to the tumour using adhesive tape so that a strong magnetic field was created in the area of the injection. This magnet was left upon the tumour for a duration of 1 h. Neither anaesthesia, nor sedation was necessary for this procedure as it was well tolerated by the cats. One week after the second i.t. gene delivery, an en bloc removal of the tumour and an entire tissue compartment was performed at the Department of Veterinary Surgery, LMU Munich. As mentioned above, control cats had their surgery scheduled without delay the day after first presentation without receiving empty plasmids. All cats had their surgery performed by the same team of surgeons and anaesthetists and were given the same anaesthesia protocol: for induction, 0.3 mg/kg BW diazepam (Diazepam®; ratiopharm, Ulm, Germany) IV or 0.2 mg/kg BW midazolam (Dormicum®; Roche, Grenzach-Wyhlen, Germany) IV, respectively, and 4.0 mg/kg BW propofol (Rapinovet®; Essex Pharma, Munich, Germany) IV; for maintenance, a gas-mixture of isoflurane (Isoba®; Essex Pharma) and Oxygen. Peri- and postoperative analgesia was ensured by the application of 0.01 mg/kg BW buprenorphine IV (Temgesic®; Essex Pharma). Intravenous fluids of 200 ml ringer lactate were given peri- and postoperatively (3.5 ml/kg BW/h) to each cat. Antibiotic treatment was started with 12.5 mg/kg BW amoxicillin-clavulanic acid (Augmentan®; GlaxoSmithKline, Munich, Germany) twice a day IV and was continued at home with the same dosage of amoxicillin-clavulanic acid (Synulox®; Pfizer, Karlsruhe, Germany) orally for five more days. For analgesia at home, cats received meloxicam (Metacam®; Boehringer Ingelheim, Ingelheim, Germany) orally in a dosage of 0.2 mg/kg BW (first day) followed by 0.1 mg/kg BW (second to fourth day).

Tumour diagnosis and histological grading

Histopathological examinations of the excised tumour specimens were performed at the Department of Veterinary Pathology, LMU, Munich. Only cats with histologically confirmed fibrosarcoma continued the study. As a consequence cats with different diagnoses were stated as drop outs and had to be replaced. This happened to cat no. 21 in dose group IV as only inflammation was diagnosed by histopathology. Furthermore, tumours of treated as well as control cats were graded histologically according to a grading pattern used by Couto et al. (2002). This pattern is based on three criteria, which are cellular morphology, presence and extension of necrosis and mitotic rate.

Follow-up

Examinations to monitor toxicity of neoadjuvant gene delivery took place on defined days. In addition to the dates on day −14 (first i.t. injection), day −7 (second i.t. injection) and day 0–2 (hospitalization for surgery) four more appointments on days 14, 45, 90 and 180 were conducted (an overview is given in Table 1). At each of these visits anamnesis was recorded, general examination was performed and blood samples for CBC and serum analyses were taken. These dates formed the observation period for possible side effects. In cases of concerns regarding general condition or evidence for recurrence owners presented their cats independent of these defined dates.

Beyond this period, two more visits on days 270 and 360 took place to search for local recurrences. The day 360 check up additionally included chest radiographs in three views and abdominal ultrasound.

Toxicity

Until day 180, the cats were evaluated for treatment-related toxicity at each of the visits. The state of health was judged by the owners' reports, physical examination, CBC and serum biochemical analyses. All occurring adverse events (AEs) were registered and graded according to the VCOG-CTCAE scale (VCOG, 2004). This is a modified CTC scale, which was adapted to veterinary medicine and contains catalogues of AEs. The grading scheme includes grade 1–5. A grade 1 AE is 'mild' whereas a grade 5 AE leads to death. Blood parameters missing in the VCOG-CTCAE scale were added according to Wieland (2002). However the grades for the parameters

Table 1. Treatment and follow-up schedule to monitor adverse events

	Day −14	Day −7	Day 0	Day 1	Day 2	Day 14	Days 45, 90 and 180
Group I–IV	Initial check up including CBC and serum analysis, first i.t. injection, MF	Clin. exam., CBC, serum analysis, second i.t. injection, MF	Preoperative check including CBC, serum analysis	Surgery	Postoperative check incl. Clin. exam., CBC	Clin. exam., Stitch removal, CBC, serum analysis	Clin. exam., CBC, serum analysis
Control group			Initial check up including CBC and serum analysis	Surgery	Postoperative check incl. Clin. exam., CBC	Clin. exam., Stitch removal, CBC, serum analysis	Clin. exam., CBC, serum analysis

Groups I–IV, treatment groups; CBC, complete blood counts; i.t., intra-tumoral; MF, magnetofection; Clin. exam., clinical examination.

monocytosis and basophilia had to be newly defined by the authors. For monocytosis, three grades were chosen: grade 1 for 500–1000 cells/μl blood, grade 2 for 1000–2000 cells/μl and grade 3 for >2000 monocytes/μl. For basophilia only two grades were defined: grade 1 for 40–100 cells/μl blood and grade 2 for >100 basophiles/μl.

Finally, for the purpose of this study a correlation between treatment and AE was formulated using a prescribed grading scheme with five steps: a grade 1 AE is certainly not connected to treatment, grades 2/3/4 mean that the AE is probably not/perhaps/probably related and a grade 5 AE is undoubtedly related to treatment. This grading scheme is subjective but this is the specified nomenclature for the evaluation of AEs.

ELISA analysis

As a part of toxicity monitoring, plasma samples were tested for feGM-CSF and feIFN-γ. Gene expression ought to be locally restricted as artificial systemic cytokine levels can provoke severe side effects, such as flu-like symptoms or the capillary leakage syndrome (Elmslie et al., 1991).

Plasma levels of feGM-CSF and feIFN-γ were measured using standard ELISA kits according to the manufacturer's instructions (DuoSet® ELISA Development System, feline GM-CSF and feline IFN-γ; R&D Systems, Minneapolis, MN, USA). Samples from days -14, -7 (taken before first/second i.t. injection), 0, 2 and 14 were measured. Both of the ELISA kits were proven to be specific by valid measurement of negative (foetal calf serum, reagent diluents and DMEM) and positive controls (supernatants from feGM-CSF/feIFN-γ producing cell culture). At the time of measurement, these kits were the only commercially available standard ELISA kits for feGM-CSF and feIFN-γ. To the best of our knowledge, there is no commercially available feIL-2 ELISA so that plasma levels of this cytokine were not measured.

Statistical analysis

Statistical analyses of the parameters white blood cells (WBC), lymphocytes, monocytes, neutrophiles, eosinophiles, basophiles and body weight were carried out by the Department of Statistics of the LMU Munich. The aim was to show whether there was a statistically significant difference regarding these parameters between the four treatment groups themselves and between treated and control cats. Tests included one-way ANOVA with appropriate *post hoc* tests (Dunnett) and Kruskal–Wallis tests. The analysis was performed with SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). A P-value of <0.05 was considered significant.

Results

Patient characteristics

A detailed overview of the 25 participating cats is given in Table 2. The control group as well as the dose groups consisted of four cats each. Only group IV was added on to eight cats as signs of treatment related toxicity occurred in cat no. 16. Cat no. 21 dropped out immediately after surgery as FSA was not confirmed by histopathology. Fourteen cats (56%) were males, 11 cats (44%) were females, all of them neutered. The mean age was 10.4 years, ranging from 4 to 15 years. Twenty-one patients were Domestic Shorthair cats (84%), the remaining four cats consisted of one Persian and three mixed breeds. Seventeen patients (68%) had primary tumours, eight (32%) had recurrences, seven of those were first recurrences, one was already the third relapse. Four tumours were assigned to stage I, three to stage II and 17 to stage III. The histological grades of the fibrosarcomas ranged from grade 1 to grade 3. There was no significant difference between tumours of control cats and those of treated cats which is not surprising as fibrosarcomas are very heterogeneous tumours. Ten of the tumours (40%) were located in the inter-scapular region, the rest (60%) was found at the lateral thoracic or abdominal wall. All of these locations serve as injection or vaccination sites in Germany. One of the cats (no. 8) had never been vaccinated but had received antibiotic injections some years ago. All of the other cats were vaccinated against feline panleucopenia and feline herpes- and calicivirus infections, 15 cats had received rabies vaccinations and 11 cats had received FeLV vaccinations. During the neoadjuvant treatment period, the measurements of the tumours did not reveal a significant shrinkage. In some of the tumours, a change regarding height could be observed on day -7 (day of second treatment): possibly as a sign of immunological reaction, these tumours appeared raised.

Owner-reported toxicity

Owner-reported toxicity included anorexia, vomiting, diarrhoea and lethargy. In all cases except for two, the reported AEs resolved without medical intervention. Cat no. 1 (group I) was hospitalized because of persisting moderate vomiting (VCOG-CTCAE-grade 2) and severe anorexia (grade 3) on day 39 after surgery. Cat no. 15 (control group) was hospitalized because of moderate vomiting and anorexia (grade 2) on day 7 after surgery. None of the participating cats had to be hospitalized during the 2 weeks of neoadjuvant treatment. One cat (no. 16, group IV) suffered from grade 3 anorexia and grade 2 vomiting after the first intra-tumoral injection. As these symptoms resolved the owners did not call for symptomatical treatment.

Weight loss

Cats were weighed at each visit in the clinic. Nineteen of 25 cats (76%) had weight loss during the treatment and follow-up period; treated cats as well as control cats. Only three cats (no. 1 of group I, no. 13 of group III and no. 16 of group IV) had mild-to-moderate weight loss (grade 1–2, maximum 11% in cat no. 13) during the 2 weeks treatment period before surgery. Medical intervention was not indicated at that time.

Haematology/clinical chemistry

At each recheck, CBC and serum biochemical analyses were performed. Two cats (no. 6 out of group II and no. 18 out of group IV) developed mild chronic renal insufficiency 6 weeks after surgery with serum creatinine concentrations close to the upper reference range at each of the following visits. Cat no. 15 (control group) had to be hospitalized 7 days after surgery

Table 2. Patient characteristics ($n = 25$)

Cat no.	Breed	Age (years)	Sex	PT/Rec.	Tumour localization	No. tum.	Tum. stage	Histol.grade
1	DSH	8	M	PT	Thoracic wall	1	II	1
2	DSH	7	M	PT	Thoracic wall	1	I	1
3	DSH	8	F	PT	Inter-scapular	1	III	2
4	DSH	9	F	PT	Inter-scapular	1	III	2
5	DSH	10	F	1st rec.	Thoracic wall	1	III	3
6	DSH	10	F	1st rec.	Thoracic wall	1	I	1
7	DSH	14	F	1st rec.	Thoracic wall	7	III	2
8	DSH	10	M	PT	Abdominal wall	1	III	3
9	DSH	14	M	1st rec.	Thoracic wall	2	III	2
10	DSH	11	F	PT	Inter-scapular	1	III	2
11	DSH	9	F	PT	Inter-scapular	1	III	3
12	DSH	7	M	PT	Thoracic wall	1	III	3
13	P	15	M	PT	Thoracic wall	1	III	3
14	DSH	14	M	PT	Inter-scapular	1	II	1
15	DSH	4	M	PT	Inter-scapular	1	I	1
16	DSH	12	F	1st rec.	Thoracic wall	2	III	2
17	DSH /A	10	M	PT	Abdominal wall	2	III	3
18	DSH	11	F	1st rec.	Thoracic wall	2	III	2
19	DSH	9	M	3rd rec.	Thoracic wall	1	I	3
20	P/NFC	11	M	PT	Inter-scapular	1	III	3
21	DSH	12	M	1st rec.	Inter-scapular	1	–	–
22	DSH	14	F	PT	Inter-scapular	1	III	3
23	TV/DSH	5	M	PT	Thoracic wall	1	II	2
24	DSH	13	M	PT	Inter-scapular	1	III	2
25	DSH	14	F	PT	Abdominal wall	2	III	2

Cat no.	Tumour volume(cm^3)	Treatment group	Evidence for rec.	Time of follow-up (days)	Drop out
1	2.09	I	No	47	Hepatolipidosis†
2	0.13	I	No	360	–
3	9.16	I	No	360	–
4	5.23	I	Yes	360	Local Rec. and l.m.
5	12.82	Control	No	360	–
6	0.36	II	No	360	–
7	5.89	II	No	360	–
8	1.33	II	Yes	210	Local Rec.
9	0.49	II	No	180	Aortic thrombosis†
10	30.89	Control	No	360	–
11	16.12	III	No	360	–
12	2.04	III	No	150	FIP†
13	29.02	III	No	240	Neurological symptoms†
14	0.40	III	No	360	–
15	0.75	Control	No	360	–
16	2.05	IV	Yes	90	Local Rec.
17	0.22	IV	Yes	140	Local Rec.
18	0.35	IV	No	360	–
19	0.01	IV	Yes	90	Local Rec.
20	13.40	Control	No	146	Accident†
21	0.01	IV	–	–	Inflammation diagnosed
22	9.67	IV	No	140	L.m.†
23	2.07	IV	No	360	–
24	2.09	IV	No	360	–
25	2.65	IV	Yes	45	Local Rec.

PT, primary tumour; Rec., recurrence; DSH, domestic shorthair; P, Persian; A, Angora; NFC, Norwegian forest Cat; TV, Turkish van; tum., tumour/s; histol., histological; FIP, feline infectious peritonitis; L.m., lung metastases; †dead.

because of life-threatening acute renal failure (serum creatinine: 1062 μmol/l, grade 4). After 5 days of intravenous fluid administration of 0.9% saline, it could be discharged with serum creatinine levels stabilized in the reference range. Cat no. 1 (group I) had to be treated in the clinic from day 39 after surgery onwards. It developed a severe liver dysfunction with increased levels of alkaline phosphatase (AP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin as well as progressing renal failure. A severe hepatolipidosis was diagnosed by fine needle aspiration. This cat died on day 47 after 9 days of intensive care. Analysis of its past medical history showed that hepatolipidosis had already been diagnosed and treated 4 years ago and food intake had always been reduced since then.

AEs regarding CBC included low haemoglobin level, low packed cell volume (PCV), leucopenia, thrombocytopenia, thrombocytosis, lymphopenia, lymphocytosis, neutropenia, left shift, monocytosis, eosinophilia and basophilia.

After analysis of all AEs, dose IV (450 μg/plasmid) is regarded to be safe and suitable for further use.

An overview of all AEs during the preoperative period is given in Table 3.

Table 3. Number of adverse events during the neoadjuvant treatment period

	Day −14 (prior to first treatment)					Day −7 (prior to second treatment)					Day 0				
	Group					Group					Group				
Adverse event (AE)	C	I	II	III	IV	C	I	II	III	IV	C	I	II	III	IV
PCV ↓	–		1		1	–		1		1			1	1	1
Leucopenia	–	1			2	–	2		1	3	2	2		1	3
Thrombocytopenia	–					–							1		
Lymphopenia	–		1		2	–		2		2	1		1	1	3
Lymphocytosis	–					–			1						
Neutropenia	–				1	–						1			1
Monocytosis	–			1	2	–							2		
Eosinophilia	–		1	2	4	–		1	1		1			1	1
Basophilia	–		1	1		–				1		1	3	1	1
AP ↑	–	1				–	1					1			
Bilirubin ↑	–					–					1				
Creatinine ↑	–	1	1			–	1	1					1		
Lethargy/fatigue	–					–	2	1	1			1	1		1
Anorexia	–	1				–	1		1	1			1	1	
Vomitus	–				1	–				2		1			
Weight loss	–				1	–			1	1				1	1

C, control group without pre-treatment, $n = 4$; Group I: 15 μg/plasmid, $n = 4$; Group II: 50 μg/plasmid, $n = 4$; Group III: 150 μg/plasmid, $n = 4$; Group IV: 450 μg/plasmid, $n = 9$ (including cat no. 21); AP, alkaline phosphatase.

Systemically detected feline cytokines

According to the standard curves plasma feGM-CSF concentrations between 0.06 and 1.13 μg/ml and plasma feIFN-γ concentrations between 0.07 and 2.66 μg/ml could be measured.

Five cats whose cytokine levels were below the detection limit on day −14 showed detectable plasma concentrations of feGM-CSF or feIFN-γ after i.t. treatment [cat no. 3 of group I, no. 15 of the control group (in this case day −14 corresponds to day 0 and this cat was not pre-treated) and nos 17, 18 and 22 of group IV]. As cytokine plasma concentrations of 11 cats were clearly above the detection limit before the first i.t. treatment and extended the entire measurement range, these concentrations had to be stated as physiological. These 11 cats belonged to all dose groups as well as to the control cats that did not receive i.t. injections. These plasma concentrations stayed constant in most cases during the treatment period.

Statistical analysis

Regarding the small group sizes, it was difficult to perform a statistical analysis. Significant differences were found on day 90 between group III and the control cats regarding the eosinophiles: The mean amount of eosinophiles was 470/μl in the control group and 142.5/μl in group III (one-way ANOVA, $P = 0.018$; *post hoc* test, $P = 0.028$). On day 0, there was a statistically significant difference between groups II and IV regarding the monocytes: mean amount 402.5/μl (group II) versus 98.75/μl (group IV) (one-way ANOVA, $P = 0.02$; *post hoc* test, $P = 0.013$). Furthermore there was a significant difference regarding the monocytes between group II (mean amount 725/μl) and control cats (mean amount 112.5/μl) on day 2 (one-way ANOVA, $P = 0.021$; *post hoc* test, $P = 0.032$).

Preliminary recurrence rates

Evaluation of recurrence rates was not the aim of this study but will be the primary aim of a subsequent phase-II trial including a larger number of patients. Nevertheless an overview of the recurrences and their time points of appearance are given in Table 2. As shown in Table 2, five cats dropped out because of treatment-unrelated deaths (no. 1: hepatolipidosis, no. 9: recurrent aortic thrombosis, no. 12: FIP, no. 13: progressing neurological symptoms, no. 20: accident). As a consequence, it was not possible to evaluate their recurrence rate 1 year after surgery. Unfortunately, autopsy was not performed on any of these cats as the owners did not agree, so that tumour-related death cannot be definitely ruled out especially in cat no. 13, which was killed because of progressing neurological symptoms.

Four of the six cats developing local recurrences belonged to group IV receiving the highest plasmid dose. In these cases tumour-free intervals ranged from 45 to 140 days only (mean 91 days).

Discussion

In Germany the standard therapy for feline FSA is only surgery as there is just one veterinary clinic with a radiotherapy facility. For this reason and because of the disadvantages of radiation therapy (full anaesthesia necessary several times, high costs for the owners, relapse in every second cat) the authors wanted to make an approach into a new therapy direction by using neoadjuvant cytokine gene transfer.

Magnetofection as a non-viral gene transfer technology was chosen for several reasons. Non-viral vectors have a much smaller biological risk than viral vectors and they are more suitable for application in veterinary clinical practice as legal regulations are not that strict. Their production is simple and affordable so that potential future demands on the medical market can easily be satisfied. Furthermore, host immunogenicity is low so that side effects caused by immune reactions against viral vectors are not expected (Niidome and Huang, 2002; Gardlik et al., 2005). The disadvantage of naked plasmid DNA is their often low efficiency in transfection. This was intended to be overcome by the use of magnetofection (Plank

et al., 2003). By that the plasmids can be focussed at a designated site (the tumour). Different studies have shown the advantages of magnetofection *in vitro* and *in vivo*: Highly increased transfection efficiency (*in vitro* a 360-fold increase in transfection efficiency of a luciferase transporter gene has been shown with magnetofection compared with conventional transfection systems) as well as a shorter time period necessary for transfection. As a consequence, dose–response profiles are greatly improved so that transfection-associated toxicity is kept low (Scherer et al., 2002; Krotz et al., 2003). It is described that CMV promoters, which are part of the plasmid construct used here, can be inhibited by IFN-γ due to its antiviral activities (Harms and Splitter, 1995). As a consequence the expression of IFN-γ as well as the expression of other cytokines can be down-regulated. However, former studies at our clinic have shown high transfection levels of IL-2 even causing fatal side effects although a CMV promoter and IFN-γ were used (Müller-Heidelberg, 2003; Wiedmann, 2005). That is why it was not considered necessary to change to a different promoter.

Intra-tumoral injections were chosen as they have been shown to be more efficient than peri-tumoral injections regarding recombinant IL-2 (Jacobs et al., 2005).

The aim of the underlying dose-escalation study was to establish a safe dose of the magnetic plasmid formulation which is well tolerated and thus can be safely used in a subsequent phase-II trial. Toxicity in participating cats remained mild-to-moderate (grade 1–2) in most cases. Six cats (no. 1, 7, 11, 15, 16 and 17) had AEs with toxicity grades 3 (severe AE) or more. In five of these cats, no correlation to neoadjuvant treatment was seen as the AEs were obviously linked to other events (no. 1: hepatolipidosis; no. 7: disturbance in wound healing; no. 11: disappearance for several weeks; no. 15: control cat; no. 17: cyclic lymphopenia from the beginning on). Only toxicity of cat no. 16 (group IV) was probably related to neoadjuvant treatment (correlation grade 4): it showed a period of vomiting and anorexia after the first i.t. injection, although it was an indoor-cat and the owners did not report any special features. This AE was self-limiting and did not recur after the second i.t. injection. For a more representative evaluation of dose group IV regarding toxicity, four more cats were added to this treatment group. As none of these cats showed any grade 3 AEs or any AEs that could be correlated to treatment, dose IV (450 μg/plasmid) was determined to be safe enough for application in subsequent studies. As the observed AEs were rather unspecific, we cannot determine which of the three cytokines provoked them. However a correct attribution is not the most important fact as a good tolerance of the cytokine combination is mainly relevant in this case.

Results of the ELISAs were partly unexpected. Before the first i.t. treatment, altogether 11 cats already had measurable amounts of plasma feGM-CSF/feIFN-γ which were clearly above the detection limit. As a consequence, these concentrations must be stated as physiological in those cats. Therefore, the measured cytokine levels of five cats after gene transfer cannot be judged as toxicity but should be regarded carefully especially as three of these cats belonged to the highest dose group (group IV). The aim of the neoadjuvant gene transfer was merely a local production of cytokines which can induce a systemic anti-tumoral immunity (Maass et al., 1995). A rise of systemic cytokine levels should be avoided because of the known associated side effects. Furthermore Zatloukal et al. (1995) achieved a better protective T-cell immune response with high localized levels of IL-2 than with systemic levels.

Due to the small group sizes, the statistical analysis lacks expressiveness and its interpretation is difficult. The significant difference on day 0 between groups II and IV regarding the monocyte count might be a result of gene transfer. Compared with group II, the amount of monocytes of group IV declined between day −7 and day 0. This might be an indication that dose IV was so high that it was able to provoke an inhibition rather than a stimulation of the immune system. The other significant results on day 2 between group II and the control cats regarding the monocytes as well as on day 90 between group III and the control cats regarding the eosinophiles cannot be reasonably explained.

A shortcoming of the described study is the lack of evidence of transfection. It is difficult to prove the transfection as there are very few possibilities to detect feGM-CSF, feIL-2 and feIFN-γ and as commercially available test kits do not seem to be very sensitive. Furthermore, the study protocol did not include biopsies of the tumours. We would have expected higher cytokine levels the day after i.t. injection than 2 weeks after the first injection when the tumour was surgically removed. To this time point, most of the cytokines would already have been decomposed by the body. Moreover, the cytokine genes were not marked with special marker proteins as their biological activity is uncertain. As a consequence, endogenous and exogenous cytokines cannot be distinguished. This shortcoming has to be considered in following studies. Taking biopsies the day after i.t. injection could be helpful for proving transfection as high levels of the feline cytokines are expected in the tissue samples.

Testing the efficacy of neoadjuvant gene transfer was not the primary aim of this dose-escalation study. Nevertheless interesting preliminary results regarding the recurrence rates could be observed and should be taken into consideration when choosing the optimal dose for a following phase-II trial. Despite the small group sizes, a tendency of early recurrences could be seen in group IV receiving the highest dose. It is known that biological agents need a defined dose window to work properly. Especially for IL-2 a bell-shaped dose dependence is described: Higher doses of this cytokine are less efficient than lower doses (Schmidt et al., 1995; Kircheis et al., 1998). This phenomenon cannot be clearly explained. Schmidt et al. presume that either the migration of antigen-loaded antigen-presenting cells into the draining lymph node or T-cell priming in the lymph nodes may be disrupted by high levels of IL-2 (Schmidt et al., 1995). Other authors proceed from the assumption that increasing quantities of IL-2 provoke proapoptotic actions in exposed T cells so that T-cell response is terminated (Abbas et al., 2000). The assumption that dose IV may lie beyond an efficient dose window has to be clarified in a phase-II study including a more representative sample size.

Summing up, dose IV (450 μg per plasmid) was well tolerated as the only AEs occurring were self-limiting and did not recur after the second i.t. injection. Dose IV can be safely tested for its efficacy in a phase-II trial. Considering the bell-shaped dose dependence of IL-2, the incidence of local recurrences in group IV and the results of the ELISA tests, it seems reasonable to test the efficacy of the third dose (150 μg/plasmid) in a subsequent phase-II study as well.

Acknowledgement

We thank the referring veterinarians for their cooperation. Furthermore we want to thank the team of anaesthetists and surgeons of the Department of Veterinary Surgery of the LMU Munich. This study was in part funded by the Nanobiotechnology programme of the German Ministry of Education and Research, project no. 13N8186.

References

Abbas, A. K., A. H. Lichtmann, and J. S. Pober, 2000: Cytokines. In: Abbas, A. K. (ed.), Cellular and Molecular Immunology, pp. 243–274. Philadelphia, PA.

Armstrong, A. C., and R. E. Hawkins, 2001: Vaccines in oncology: background and clinical potential. Br. J. Radiol. **74,** 991–1002.

Berzofsky, J. A., M. Terabe, S. Oh, I. M. Belyakov, J. D. Ahlers, J. E. Janik, and J. C. Morris, 2004: Progress on new vaccine strategies for the immunotherapy and prevention of cancer. J. Clin. Invest. **113,** 1515–1525.

Chou, C. Y., K. F. Hsu, S. T. Wang, S. C. Huang, C. C. Tzeng, and K. E. Huang, 1997: Accuracy of three-dimensional ultrasonography in volume estimation of cervical carcinoma. Gynecol. Oncol. **66,** 89–93.

Clary, B. M., E. C. Coveney, D. G. Blazer III, R. Philip, and H. K. Lyerly, 1996: Active immunotherapy of pancreatic cancer with tumor cells genetically engineered to secrete multiple cytokines. Surgery **120,** 174–181.

Cohen, M., J. C. Wright, W. R. Brawner, A. N. Smith, R. Henderson, and E. N. Behrend, 2001: Use of surgery and electron beam irradiation, with or without chemotherapy, for treatment of vaccine-associated sarcomas in cats: 78 cases (1996-2000). J. Am. Vet. Med. Assoc. **219,** 1582–1589.

Couto, S. S., S. M. Griffey, P. C. Duarte, and B. R. Madewell, 2002: Feline vaccine-associated fibrosarcoma: morphologic distinctions. Vet. Pathol. **39,** 33–41.

Cozzi, P. J., P. A. Padrid, J. Takeda, M. L. Alegre, N. Yuhki, and A. R. Leff, 1993: Sequence and functional characterization of feline interleukin 2. Biochem. Biophys. Res. Commun. **194,** 1038–1043.

Dow, S. W., R. E. Elmslie, A. P. Willson, L. Roche, C. Gorman, and T. A. Potter, 1998: *In vivo* tumor transfection with superantigen plus cytokine genes induces tumor regression and prolongs survival in dogs with malignant melanoma. J. Clin. Invest. **101,** 2406–2414.

Elmslie, R. E., S. W. Dow, and G. K. Ogilvie, 1991: Interleukins: biological properties and therapeutic potential. J. Vet. Intern. Med. **5,** 283–293.

Gardlik, R., R. Palffy, J. Hodosy, J. Lukacs, J. Turna, and P. Celec, 2005: Vectors and delivery systems in gene therapy. Med. Sci. Monit. **11,** RA110–RA121.

Harms, J. S., and G. A. Splitter, 1995: Interferon-gamma inhibits transgene expression driven by SV40 or CMV promoters but augments expression driven by the mammalian MHC I promoter. Hum. Gene Ther. **6,** 1291–1297.

Hendrick, M. J., and M. H. Goldschmidt, 1991: Do injection site reactions induce fibrosarcomas in cats? J. Am. Vet. Med. Assoc. **199,** 968.

Hirschberger, J., and M. Kessler, 2001: Das feline Fibrosarkom. Tierarztl. Prax. **29,** 66–71.

Jacobs, J. J., D. Sparendam, and W. Den Otter, 2005: Local interleukin 2 therapy is most effective against cancer when injected intratumourally. Cancer Immunol. Immunother. **54,** 647–654.

Jourdier, T. M., C. Moste, M. C. Bonnet, F. Delisle, J. P. Tafani, P. Devauchelle, J. Tartaglia, and P. Moingeon, 2003: Local immunotherapy of spontaneous feline fibrosarcomas using recombinant poxviruses expressing interleukin 2 (IL2). Gene Ther. **10,** 2126–2132.

Kass, P. H., W. G. Barnes Jr, W. L. Spangler, B. B. Chomel, and M. R. Culbertson, 1993: Epidemiologic evidence for a causal relation between vaccination and fibrosarcoma tumorigenesis in cats. J. Am. Vet. Med. Assoc. **203,** 396–405.

Kempf, C., 2005: Nonviraler Gentransfer der felinen Zytokin-Gene IL-2, IFN-gamma und GM-CSF als adjuvante Immuntherapie beim Fibrosarkom der Katze. Eine klinische Phase I-Studie. Diss. med. vet. Ludwig Maximilian University, Munich.

Kircheis, R., Z. Kupcu, G. Wallner, and E. Wagner, 1998: Cytokine gene-modified tumor cells for prophylactic and therapeutic vaccination: IL-2, IFN-gamma, or combination IL-2 + IFN-gamma. Cytokines Cell. Mol. Ther. **4,** 95–103.

Krotz, F., H. Y. Sohn, T. Gloe, C. Plank, and U. Pohl, 2003: Magnetofection potentiates gene delivery to cultured endothelial cells. J. Vasc. Res. **40,** 425–434.

Maass, G., T. Schweighoffer, M. Berger, W. Schmidt, E. Herbst, K. Zatloukal, M. Buschle, and M. L. Birnstiel, 1995: Tumor vaccines: effects and fate of IL-2 transfected murine melanoma cells *in vivo*. Int. J. Immunopharmacol. **17,** 65–73.

Macy, D., and C. Guillermo Couto, 2001: Prevention and treatment of injection-site sarcomas. J. Feline Med. Surg. **3,** 169–170.

Müller-Heidelberg, M., 2003: Immunologisches Monitoring der Gentherapie des Fibrosarkoms der Katze, Zytokinkinetik und humorale Antwort auf Vektor und Transgen. Diss. med. vet. Ludwig Maximilian University, Munich.

Niidome, T., and L. Huang, 2002: Gene therapy progress and prospects: nonviral vectors. Gene Ther. **9,** 1647–1652.

Plank, C., U. Schillinger, F. Scherer, C. Bergemann, J. S. Remy, F. Krotz, M. Anton, J. Lausier, and J. Rosenecker, 2003: The magnetofection method: using magnetic force to enhance gene delivery. Biol. Chem. **384,** 737–747.

Quintin-Colonna, F., P. Devauchelle, D. Fradelizi, B. Mourot, T. Faure, P. Kourilsky, C. Roth, and M. Mehtali, 1996: Gene therapy of spontaneous canine melanoma and feline fibrosarcoma by intratumoral administration of histoincompatible cells expressing human interleukin-2. Gene Ther. **3,** 1104–1112.

Rosenthal, F. M., K. Cronin, R. Bannerji, D. W. Golde, and B. Gansbacher, 1994: Augmentation of antitumor immunity by tumor cells transduced with a retroviral vector carrying the interleukin-2 and interferon-gamma cDNAs. Blood **83,** 1289–1298.

Scherer, F., M. Anton, U. Schillinger, J. Henke, C. Bergemann, A. Kruger, B. Gansbacher, and C. Plank, 2002: Magnetofection: enhancing and targeting gene delivery by magnetic force *in vitro* and *in vivo*. Gene Ther. **9,** 102–109.

Schmidt, W., T. Schweighoffer, E. Herbst, G. Maass, M. Berger, F. Schilcher, G. Schaffner, and M. L. Birnstiel, 1995: Cancer vaccines: the interleukin 2 dosage effect. Proc. Natl Acad. Sci. USA **92,** 4711–4714.

VCOG, 2004: Veterinary Co-operative Oncology Group 2004 consensus statement-common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.0. Vet. Comp. Oncology **2,** 194–213.

Wiedmann, K., 2005: Klinische Phase I-Studie zur neoadjuvanten immunstimulierenden Therapie des felinen Fibrosarkoms mit Interleukin-2 und Interferon-gamma. Diss. med. vet. Ludwig Maximilian University, Munich.

Wieland, S., 2002: Klinische Phase I-Studie zur gentherapeutischen Immunstimulation durch Interleukin-2 und Interferon-gamma als adjuvante Behandlung des felinen Fibrosarkoms. Diss. med. vet. Ludwig Maximilian University, Munich.

Yoon, S. J., D. S. Heo, J. O. Kang, S. G. Lee, C. D. Kim, M. W. Sung, and N. K. Kim, 1998: Synergistic anti-tumor effects with co-expression of GM-CSF and IFN-gamma in murine tumors. Int. J. Cancer **77,** 907–912.

Zatloukal, K., A. Schneeberger, M. Berger, W. Schmidt, F. Koszik, R. Kutil, M. Cotten, E. Wagner, M. Buschle and G. Maass, 1995: Elicitation of a systemic and protective anti-melanoma immune response by an IL-2-based vaccine. Assessment of critical cellular and molecular parameters. J. Immunol. **154,** 3406–3419.