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RESEARCH ARTICLE

Neoadjuvant gene delivery of feline granulocyte-macrophage colony-stimulating factor using magnetofection for the treatment of feline fibrosarcomas: a phase I trial

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Abstract

Despite aggressive pre- or postoperative treatment, feline fibrosarcomas have high recurrence rates. Immunostimulatory gene therapy is a promising approach in veterinary oncology. This phase I dose-escalation study was performed to determine toxicity and feasibility of gene therapy with feline granulocyte-macrophage colony-stimulating factor (feGM-CSF) in cats with fibrosarcomas. Twenty cats were treated with plasmid coding for feGM-CSF attached to magnetic nanoparticles in doses of 50, 250, 750 and 1250 μg. Two preoperative intratumoral injections followed by magnetofection were given. Four control cats received only surgical treatment. Adverse events were recorded and correlated according to the veterinary co-operative oncology group toxicity scale. An enzyme-linked immunosorbent assay was performed to detect plasma feGM-CSF concentrations. No significant treatment related toxicity was observed. Preliminary recurrence results were encouraging as, on day 360, ten of 20 treated cats were recurrence-free. In conclusion, 1250 μg of feGM-CSF plasmid DNA applied by magnetofection is safe and feasible for phase II testing. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords cytokine; feGM-CSF; feline fibrosarcoma; immunostimulatory gene therapy; *in situ* vaccination; magnetofection

Introduction

The problem of an increasing incidence of reactions at sites commonly used for vaccinations and injections in cats was first recognized in the USA in 1991 [1]. Evidence for a causal relationship between vaccination and the development of soft tissue sarcomas at injection sites in cats soon accumulated [2,3]. These findings were emphasized by the identification of aluminum, which is commonly used as a vaccine adjuvant, in biopsies of cats with fibrosarcomas [4].

The exact mechanism by which vaccines can induce tumor formation is unknown, but it is known that adjuvants such as aluminum hydroxide enhance the chronic immune response. This can result in inflammatory granulomas at the site of vaccination, which may promote the neoplastic transformation of fibroblasts in predisposed cats [4,5].

Sarcomas developing at sites associated with vaccination are reported to occur in younger cats than sarcomas at other sites. They are also more aggressive, are larger at the time of diagnosis and are more likely to recur after

surgery compared to sarcomas arising at sites unassociated with vaccination [3].

The incidence of injection site sarcomas was estimated 1–3.6 per 10.000 cats for the USA [2,6]. No such estimates exist for Europe.

The metastatic rate of 10–28% of cats with fibrosarcomas is low, but these tumors tend to local recurrence in up to 70% of the cases after surgery as sole therapy [7–9]. Tumor recurrence after incomplete resection can occur as early as 2 weeks after surgery [10], but typically occurs within the first 6 months [3]. Radical surgery with wide margins can extend the tumor-free interval and the survival time, but is often difficult to manage [11].

Jourdier *et al*. [12] conducted a study where 18 cats with spontaneous fibrosarcomas received iridium-based radiotherapy following surgical removal. Eleven of these 18 cats (61%) had a recurrence within 12 months after treatment. In a retrospective study where vaccine-associated fibrosarcomas were treated with postoperative radiation alone (50 cats) or in combination with chemotherapy (26 cats), 32 of all 76 cats developed tumor relapse (41%) [13]. Another retrospective study reviewed preoperative radiotherapy in 92 cats with vaccine associated sarcomas [14]. Additional chemotherapy in 33 of these cats failed to show a significant effect.

Many more studies of applying chemotherapy for the treatment of feline fibrosarcoma have been conducted, but the results obtained were disappointing. Neither doxorubicin, liposome-encapsulated doxorubicin [15,16], the combination of doxorubicin and cyclophosphamide [17], nor the use of lomustine [18], could improve the disease-free time or overall survival satisfactorily. In a recent study, cats with locally advanced, recurrent or metastatic vaccine-associated sarcomas were treated with ifosfamide, but a response could only be achieved in 11 of 27 treated cats for a median duration of 70 days [19]. Another phase I clinical trial evaluated the toxicity of imatinib mesylate, a tyrosine kinase inhibitor, in nine cats with various tumors [20]. All four cats with vaccine-associated sarcomas responded to the treatment with imatinib, but there was only tumor stabilization for an average of 2 months. Although new treatments improved the prognosis of fibrosarcomas in cats, local recurrence is still frequently observed, and additional therapies are required to complement these current treatment options.

There has been a variety of immunological strategies, including cytokine gene transfer, to elicit anti-tumor responses to cause regression of established tumors [21–23]. In recent years, there were promising reports of direct *in vivo* transfection of tumors with cytokines as an alternative strategy with several advantages [12,24,25]. Quintin-Colonna *et al*. [26] performed a clinical trial for the treatment of feline fibrosarcoma by administering histo-incompatible cells expressing human interleukin-2 into the peritumoral area after surgery and radiotherapy. In these cats, median survival time was prolonged from 8 months to more than 16 months. These results demonstrated the safety and therapeutic potential of *ex vivo* gene transfer in veterinary patients with metastatic and non-metastatic tumors for the first time. The rationale for the use of cytokines for *in situ* vaccination is the hypothesis that the transfer of cytokine genes into tumor cells will enhance the host's immune response to both the primary tumor and distant metastases [27]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is required for the survival, proliferation and differentiation of hematopoietic progenitor cells, especially for those of the granulocyte and macrophage lineage [28]. It stimulates anti-tumor immunity by augmenting the antigen-presenting activity of macrophages [21]. In one study, the production of GM-CSF directly correlated with infiltrating macrophages and their metalloelastase activity [29]. This is considered to be another mechanism of suppression of tumor metastases by GM-CSF secreting tumor cells besides the stimulation of antigen presenting cells. In a recent study, the expression of matrix metalloproteinases in feline vaccine site-associated sarcomas was investigated and it was found that the duration of survival was affected by the expression of these endopeptidases [30].

In human and veterinary medicine, there have been promising reports of using human GM-CSF (huGM-CSF) as potent weapon in the treatment of various tumors [31,32]. As the homology between huGM-CSF and feline GM-CSF (feGM-CSF) is only 69% and the majority of cats treated with huGM-CSF develop an antibody response against the xenogenic cytokine [33], gene therapy using species-specific cytokine genes may provide further benefits for feline fibrosarcoma patients.

The principal objective of this dose-escalation study was to determine toxicity and feasibility of anti-cancer immune therapy via gene transfer of feGM-CSF in cats with fibrosarcomas. Furthermore, preliminary results for the efficacy of this neoadjuvant therapy protocol were observed and reported.

Materials and methods

Patient selection

The study was conducted as a prospective phase I dose-escalation study with four previously defined increasing doses of plasmid coding for feGM-CSF. Client-owned cats with clinical diagnosis of fibrosarcoma entered the study. Written informed consent from owners was obtained before the cats were enrolled. Patients with primary tumors as well as recurrences were accepted but both had to be located at the trunk. Additional inclusion criteria were that tumors could be removed surgically in one setting and that cats had a life expectancy of at least 1 year independent of the tumor disease. Exclusion criteria were other malignancies than fibrosarcoma at the time of presentation or in the medical history, pregnancy and metastases. Cats were also excluded if it would have been necessary to perform amputation during surgery. Furthermore, cats were not enrolled if they ever received radio-, chemo- or gene therapy in the past or if they

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had been treated with immunosuppressives during the preceding 6 weeks.

Initial evaluation

At the initial check-up, complete medical history and vaccination history was recorded. Physical examination was performed and blood samples were taken. External tumor measurements in three dimensions were recorded so that tumor volume and staging could be defined according to Chou *et al*. [34] and Hirschberger and Kessler [35]. Complete blood count (CBC) including differential blood count was performed. The recorded serum biochemistry profile included aspartate aminotransferase, alkaline phosphatase activities, blood urea nitrogen, serum creatinine concentration, total protein, albumin, sodium, potassium, calcium, phosphorus, chloride, bilirubin and glucose concentrations, as well as thyroxine concentration. In addition, a test for feline immunodeficiency virus antibodies and a feline leukaemia virus antigene test were performed. To search for metastases, thoracic radiographs (left lateral, right lateral and ventrodorsal view) and abdominal ultrasonography were conducted.

Plasmid and magnetic nanoparticles

The gene encoding feGM-CSF was isolated from feline blood cells during a previous study [36]. It was cloned into an expression plasmid under the control of the cytomegalovirus promoter. Plasmid preparations were carried out by Plasmid Factory GmbH & Co. KG (Bielefeld, Germany). Aqueous solutions of the plasmid were mixed at 1 : 1 (w/w) ratios with polyethylenimine (PEI)-coated iron oxide magnetic nanoparticles (transMAG^{PEI}; Chemicell GmbH, Berlin, Germany). The positively charged PEI coating mediates DNA binding to the magnetic nanoparticles via electrostatic interactions. The total injection volume was always 500 µl. After the intratumoral injection of this solution, a magnetic gradient field was applied to the tumor for 1 h by taping a neodymium-iron-boron magnet (Neo Delta magnet NE2010; IBS Magnet, Berlin, Germany) onto the tumor region.

Neoadjuvant treatment

Cats were treated with feGM-CSF gene therapy on day −14 (i.e. day of initial check-up) and on day −7. Before the second treatment, a clinical examination including tumor measurement and monitoring for signs of adverse events (AE) was performed. Additionally, blood samples for a CBC and a serum biochemistry profile were taken. Plasmid coding for feGM-CSF was given in increasing doses of 50, 250, 750 and 1250 µg per intratumoral injection. Such a dose escalation strategy (i.e. dose elevation by a factor of five with addition of an interims dose at the 66% level of the highest given dose) was also used in a study formerly conducted by the same working group [37]. Four cats were enrolled as control cats. For ethical reasons and the potential hazard of injection, these cats had surgery without receiving empty plasmids or placebo the day after first presentation. Each dosage was administered to four cats. Dose-escalation was performed, provided that none of the treated cats had dose-limiting toxicity (DLT). The dosage of plasmid coding for feGM-CSF recommended for phase II trials was defined as the highest dosage where no DLT could be detected. If cats in a dose group showed DLT, three additional cats were treated with the lower dose. When no DLT was observed in these three additional cats, the dosage was escalated again until the highest defined dosage (1250 µg) was reached.

Surgery and histopathology

Cats were hospitalized at the Clinic of Small Animal Medicine from day 0 to day 2. On day 0, a complete examination including tumor measurement, CBC and a serum biochemistry profile was performed and a permanent venous catheter (intravenious catheter) was placed. Cats received amoxicillin-clavulanic acid at a dosage of 12.5 mg/kg b.w., q. 12 h, i.v. (Augmentan; GlaxoSmithKline, Munich, Germany) as antibiotic treatment during their whole hospitalization. For pre- and postoperative analgesia, buprenorphin at 0.01 mg/kg b.w., q. 12 h, i.v. (Temgesic; Essex Pharma, Munich, Germany) was administered. All surgeries were performed on day 1 as *en bloc* resection by the same team of surgeons from the Clinic of Small Animal Surgery and Reproduction. The anesthesia protocol was identical in all cats: for induction, midazolam at 0.1 mg/kg b.w., i.v. (Dormicum; Roche, Grenzach-Wyhlen, Germany) or diazepam at 0.3 mg/kg b.w., i.v. (Diazepam; Ratiopharm, Ulm, Germany) and propofol at 4.0 mg/kg b.w., i.v. (Rapinovet; Essex Pharma). For maintenance isoflurane (Isoba; Essex Pharma) and oxygen were given per inhalationem. After the excision, every tumor was sent to the Department of Veterinary Pathology of the LMU Munich for routine histopathological evaluation. Only cats with histologically confirmed fibrosarcoma continued the study. On day 2, cats were examined and blood samples for CBC were taken. If cats were in good general condition and blood work was without severe abnormalities, cats were discharged. For postoperative analgesia cats received meloxicam (Metacam; Boehringer Ingelheim, Ingelheim, Germany) at a dosage of 0.2 mg/kg b.w., q. 24 h, per os (first day) and 0.1 mg/kg b.w., q. 24 h, per os for an additional 3 days. For continuative antibiotic treatment, cats obtained amoxicillin-clavulanic acid (Synulox; Pfizer, Karlsruhe, Germany) at a dosage of 12.5 mg/kg b.w., q. 12 h, per os for a period of 5 days. At the follow-up evaluation on day 14, the stitches were taken out.

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Follow-up evaluations to monitor adverse events

The follow-up schedule to monitor toxicity in the form of AEs included routine visits on days 14, 45, 90 and 180 after surgery. Unscheduled visits were conducted when the owners reported suspicion or evidence of AEs or tumor relapse. A clinical examination including observation of wound healing and a clinical check for recurrences by palpation of the tumor excision site and the lymph nodes was performed during each presentation. Furthermore, a laboratory profile including CBC and a serum biochemistry profile were performed at each routine visit. Two more visits on day 270 and 360 were scheduled to obtain preliminary recurrence results. At the final visit on day 360, thoracic radiographs (left lateral, right lateral and ventrodorsal view) and abdominal ultrasonography are additionally conducted.

Common terminology criteria for adverse events (CTCAE)

All findings of the study were documented detailed in case report forms. Any AE, such as vomiting, diarrhea, lethargy or anorexia as well as any clinically relevant increase or decrease of a hematological or biochemical laboratory parameter were documented. AEs from day −14 until day 180 were recorded according to the veterinary co-operative oncology group toxicity scale (VCOG-CTCAE) [38]. AEs missing in this scheme were defined by the authors (Table 1). To assess whether the observed AEs could be correlated with the neoadjuvant therapy, attribution was assigned for each AE, using the correlation grades (CG): 'definite (CG 5)' means the AE is clearly related to the intervention; 'probable (CG 4)' is used when the AE is likely related; 'possible (CG 3)' when it may be related; 'unlikely (CG 2)' if the AE is doubtfully related; and 'unrelated (CG 1)' if the AE is clearly not related to the neoadjuvant therapy.

Statistical analysis

Statistical analysis was performed by the Institute for Statistics for changes within the parameters body weight, white blood cells, monocytes, lymphocytes, neutrophils, eosinophils and basophils. All values of these parameters were analyzed from day −14 until day 90 and were compared within the dose groups as well as between the dose groups and controls. Tests included one-way analysis of variance with appropriate post-hoc tests (Dunnett) and Kruskall–Wallis tests. $p < 0.05$ was considered statistically significant. Analyses were performed with statistical software [39].

Enzyme-linked immunosorbent assay (ELISA)

To determine systemic levels of feGM-CSF, an ELISA (DuoSet ELISA Development System, feline GM-CSF; R&D Systems, Minneapolis, MN, USA) was performed according to the manufacturer's instructions, using the plasma samples of study cats from days −14, −7, 0, 2 and 14. For ELISA kit validation, ELISAs were performed with supernatants from cultured cells transfected with the feline GM-CSF gene by magnetofection.

To verify that the intratumoral cytokine gene vaccination actually results in the expression of transfected cytokine genes in patient tumors, seven patients were treated with 1250 µg of plasmid DNA coding for the human GM-CSF (huGM-CSF) gene following the magnetofection protocol described above (the huGM-CSF gene was cloned into the same plasmid backbone under the same promoter as the feGM-CSF gene). This was performed because the problem of distinguishing endogenous feGM-CSF and expression of the transfected feGM-CSF gene prevails. In detail, two cats were magnetofected with one dose 1 day prior to surgery (cat nos. I and II), two cats were treated with two doses 14 days and 7 days prior to surgery (cat nos VI and VII), and three cats were treated with only one dose 7 days prior to surgery (cat nos III, IV and V). This schedule was carried out because the expression kinetics of transfected genes in patient tumors were unknown. The surgically removed tumors were cut into pieces of approximately 3–5 mm × 3–5 mm. Representative samples from various tumor regions were distributed in a random fashion to several 3-cm culture dishes and incubated at 37 °C/5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Biochrom AG, Berlin, Germany) supplemented with 10% fetal calf serum (PAN; Biotech GmbH, Aidenbach, Germany), 500 U penicillin/500 ml, as well as 50 mg streptomycin/500 ml

Table 1. Criteria for grading of hematologic toxicity in addition to VCOG-CTCAE[a]

Adverse event	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Basophilia	0–100/µl	>100/µl	–	–	–
Eosinophilia	>600–3000/µl	>3000/µl	–	–	–
Left shift	>600–3000/µl	>3000/µl	–	–	–
Leukopenia	4500– < 6000/µl	2000– < 4500/µl	500– < 2000/µl	<500/µl	–
Lymphocytopenia	800– < 1000/µl	500– < 800/µl	<500/µl	–	–
Lymphocytosis	>4000–10 000/µl	>10 000/µl	–	–	–
Monocytosis	0–1000/µl	>1000–2000/µl	>2000/µl	–	–
Thrombocytosis	>55 0000–1000 000/µl	>1000 000/µl	–	–	–

–, Not defined. [a]VCOG-CTCAE [38].

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(Biochrom AG). After 24 h, supernatants from all cultures were taken for analysis for huGM-CSF expression by ELISA which was carried out according to the instructions of the manufacturer (Biotrak; GE Healthcare, Chalfont St Giles, Great Britain). To rule out cross-reactivity between feline and human GM-CSF, supernatants of tumor cells from study cats treated with feGM-CSF that were positive in a feGM-CSF ELISA (DuoSet ELISA Development System, feline GM-CSF; R&D Systems) were used as a negative control. Additionally, supernatants of tumors from control cats (untreated) were used as negative controls.

Results

Patient characteristics

Between February 2005 and March 2006, 29 cats with clinical diagnosis of fibrosarcoma entered the phase I trial and were treated with feGM-CSF as gene therapy at four dose levels or were enrolled as control cats. The cats comprised 25 domestic short hair cats, one Norwegian Forest cat, one Maine Coon cat, one Oriental Shorthair and one Norwegian Forest cat-mix. There were 13 neutered females and 16 neutered males. The ages of the cats were in the range 4–16 years (mean = 9.8 years; median = 11.0 years). Nineteen cats had primary tumors, ten cats had recurring tumors, of which two already had the second recurrence. Tumors were predominantly located in the scapular region ($n = 15$) and at the thoracic wall ($n = 10$). Other sites were the cervical ($n = 2$) and the abdominal region ($n = 2$). All tumors were located at presumed injection sites. Four cats had tumor stage I (tumor size <2 cm), eight cats had tumor stage II (tumor size 2–3 cm) and 17 cats had tumor stage III (tumor size >3 cm or multiple tumors).

All surgeries were performed as *en bloc* resection with margins of 3 cm whenever possible. Macroscopically, all tumors were excised with margins of healthy tissue. The histopathological evaluation of the tumors revealed 24 fibrosarcomas, one panniculitis and one calcinosis circumscripta.

Treatment groups

As shown in Table 2, four cats received the first dose of 50 μg plasmid coding for feGM-CSF. As none of these cats showed any severe AE, the higher dose of 250 μg was administered to the subsequent four cats. As the first cat (no. 10) receiving the third dose of 750 μg plasmid coding for feGM-CSF died 6 days after the first injection, three more cats had to be treated with the second dose. Cat no. 11 died at induction of anesthesia, so this cat had to be replaced to complete the second dose group. These additional three cats showed no signs of toxicity, so the dose of plasmid coding for feGM-CSF was again elevated to 750 μg and further to 1250 μg. The four cats receiving the highest dose showed no severe signs of hematological and gastrointestinal toxicity but three of them showed owner reported anorexia, lethargy or vomiting on day −7 or 0; thus, two additional cats were enrolled in this dose group to characterize toxicity further on. The additionally enrolled cats did not show any signs of toxicity. Four cats were enrolled as the control group. For ethical reasons, control cats had their surgery without receiving placebo the day after first presentation.

Adverse events

All AEs are summarized in Table 3. For each dose level and each study period, the amount of AEs are given.

Adverse events: gastrointestinal and owner-reported parameters

Control group: Cat no. 16 was presented on day 7 after surgery with clinical signs of anorexia (grade 2), dehydration (grade 2) and vomiting (grade 2). Serum biochemical analysis revealed life threatening azotaemia (grade 4) so that the cat was hospitalized and intensively treated with intravenous fluids. After 5 days, serum creatinine concentration and blood urea levels were within the reference range and the cat was discharged.

Dose group 1 (50 μg feGM-CSF): In this group, only mild, self-limiting AEs (grade 1) occurred

Dose group 2 (250 μg feGM-CSF): Cat no. 11 showed no abnormal findings at the check-up and no reported signs of AEs after the injections, but died at induction of anesthesia. After administering diazepam and propofol intravenously, the cat showed apnea (grade 5) and subsequently cardiac arrest. Despite intubation, reanimation and intracardiac epinephrine injections the cat died. The owner consented to an autopsy, which revealed the possibility of a reduced anesthesia tolerance because of a restrictive cardiomyopathy. The owner of cat no. 13 reported a concomitant episode of indigestion with mild anorexia (grade 1) at the day of the initial check-up, which resolved by day −7. On day −1, the owners presented their cat to the referring veterinarian because of vomiting (grade 3) and recurring anorexia (grade 3). The veterinarian treated the cat with intravenous fluids, antibiotics and metoclopramide. After 4 days the food intake normalized but, 3 days later, the cat was hospitalized because of recurring anorexia and vomitus. The cat was medicated for gastritis and a gastroduodenoscopy was performed during the same anesthesia as surgery of the fibrosarcoma. Macroscopically, there were no pathological findings and the histopathological evaluation of samples taken during endoscopy did not reveal any pathological findings. The cat showed neither anorexia, nor vomiting after surgery

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Table 2. Patient characteristics and preliminary recurrence results

Cat no.	feGM-CSF dose (μg)	Breed	Age	Sex	Primary tumor	Tumor volume (cm^3)	Region of tumor localisation	Tumor stage	Evidence for local recurrenc	Evidence for metastases	Last day of follow-up
1	50	DSH	7	nm	Yes	1.0	Scapular	II	Yes	No	270
2	50	Norw. Forest cat	9	nf	No	0.1	Thoracic	III	No	No	360
3	50	DSH	7	nf	Yes	4.1	Scapular	– [a]	– [a]	– [a]	– [a]
4	0	DSH	10	nf	No	12.7	Thoracic	III	No	No	360
5	50	DSH	5	nm	No	0.3	Interscapular	III	Yes	No	45
6	250	DSH	9	nm	Yes	0.2	Abdominal	II	Yes	No	180
7	250	DSH	11	nm	Yes	0.4	Thoracic	II	No	No	360
8	250	DSH	12	nf	Yes	10.2	Thoracic	III	Yes	No	180
9	250	DSH	10	nm	Yes	9.4	Cervical	III	No	Yes	300
10	750	DSH	13	nm	Yes	12.4	Interscapular	III	– [b]	– [b]	– [b]
11	250	DSH	7	nf	No[1]	0.2	Thoracic	I	– [c]	– [c]	– [c]
12	250	DSH	7	nm	Yes	27.6	Thoracic	III	No	No	360
13	250	DSH	8	nf	No	30.3	Scapular	III	Yes	No	45
14	250	DSH	7	nf	Yes	13.0	Abdominal	III	Yes	Yes	170
15	0	DSH	11	nf	Yes	30.7	Scapular	III	No	No	360
16	0	DSH	4	nm	Yes	0.8	Scapular	I	No	No	360
17	750	DSH	12	nm	Yes	1.7	Thoracic	II	No	No	360
18	750	Oriental Shorthair	12	nf	Yes	0.5	Interscapular	– [d]	– [d]	– [d]	– [d]
19	750	DSH	6	nf	Yes	1.6	Interscapular	II	No	No	360
20	750	DSH	11	nm	No	7.8	Interscapular	III	Yes	No	325
21	750	DSH	11	nf	No[2]	24.2	Cervical	III	Yes	No	300
22	0	Norw. Forest cat	11	nm	Yes	13.3	Scapular	III	No	No	150[e]
23	1250	Maine Coon cat	16	nf	Yes	2.8	Scapular	II	No	No	360
24	1250	DSH	13	nm	Yes	24.1	Thoracic	III	No	Yes	150
25	1250	DSH	11	nf	Yes	1.4	Scapular	II	No	No	360
26	1250	DSH	12	nm	No	14.2	Thoracic	III	– [f]	– [f]	– [f]
27	1250	DSH	12	nm	Yes	19.7	Interscapular	III	No	No	360
28	1250	DSH	11	nf	No	0.6	Thoracic	III	No	No	360
29	1250	DSH	9	nm	No	0.1	Scapular	I	No	No	360

feGM-CSF, feline granulocyte-macrophage colony-stimulating factor; DSH, domestic shorthair cat; nm, neutered male; nf, neutered female; [1,2]second recurrence. [a]Pathohistological diagnosis was panniculitis; [b]died at day −8; [c]died at induction of anesthesia; [d]Pathohistological diagnosis was calcinosis circumscripta; [e]died in consequence of an accident at day 150; [f]no owner-compliance, end of study at day −13.

and was discharged with a medication of antibiotics, metoclopramide and sucralfate for another week.

Dose group 3 (750 μg feGM-CSF): On day −8, cat no. 10 was found dead after an episode of anorexia of three days (grade 5). Autopsy revealed pre-existing cardiac and renal alterations, and necrosis of the crypt epithelial cells of the small intestine and the epithelial cells of the plicae aryepiglotticae with infiltration of neutrophils. According to the pathologist, the findings at the intestine and the larynx were consistent with a hyperacute course of feline panleukopenia.

Dose group 4 (1250 μg feGM-CSF): The owner of cat no. 23 reported that the cat had shown lethargy (grade 1) and anorexia (grade 3) for a period of 2 days, approximately 3 days after the first injection. On clinical examination on day −7, the cat had lost 250 g in weight. After the second injection, food intake was unaltered again and the weight stayed constant. The owner of cat no. 24 reported lethargy (grade 1) on day −7 and on day 0. On day 5 after surgery, this cat showed deteriorative lethargy (grade 2), vomitus (grade 2), dehydration (grade 2) and anorexia (grade 3) with a weight loss of 200 g and was therefore presented and hospitalized on day 9. After treatment with intravenous fluids, ranitidine and diazepam to raise the appetite, the cat was discharged the next day with recovered food intake.

Gastrointestinal and owner-reported events that can be correlated as possibly related (CG 3) to the neoadjuvant gene therapy occurred in cats of all dose groups. Only one cat (no. 24) showing these AEs had to be treated and hospitalized for one night. All other possibly related adverse events were self-limiting.

Adverse events: hematologic parameters

No toxicity of grade 4 or more was detected. Several cats of all dose groups showed decreased hemoglobin and packed cell volume (PCV) levels on days 2 and 14 (grades 1, 2 and 3) because of blood loss during surgery. Changes in white blood count and differential blood count are shown in Table 3. These changes occurred in cats of all dose groups (treated as well as control cats) and at each time the blood samples were taken.

Adverse events: constitutional parameters

Weight loss (grade 1 and 2) was detected in many cats from day 14 after surgery onward. Cats of all dose groups, including the control group, were affected and most of them did not regain their weight by day 180.

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Table 3. Summary of adverse events. Each number in a row is representing the number of adverse events occurring in the particular observation period

	Time of diagnosis and first injection (day −14)																				Second injection (day −7)																	
	Dose 1					Dose 2					Dose 3					Dose 4					Dose 1					Dose 2					Dose 3					Dose 4				
Grade	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Hemoglobin											1																												
PCV											1																												
Thrombocytopenia						1										1															1								
Thrombocytosis																																						
Leucopenia	1					3					1					2					1	1				4					1					1	1			
Neutropenia	1																				1																		
Monocytosis						1																				1													
Lymphopenia												1	1					4									1				1					3	1			
Lymphocytosis						1					1																				1								
Left shift																																						
Eosinophilia	1					1					2															1					1								
Basophilia																1															1								
Lethargy																										1					1					2			
Weight loss																																				1				
Anorexia						1																1					1			1	1		1					
Dehydration						1																																	
Diarrhea																																							
Vomiting																					1										1					1				
Albumin low																																							
AST																1																							
Calcium low																1																							
Creatinine						1															1					1													
Apnea																																							

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Table 3. (***Continued***)

	Pre-operative (day 0)																										Post-operative (day 2)																					
	Control					Dose 1					Dose 2					Dose 3					Dose 4					Control					Dose 1					Dose 2					Dose 3					Dose 4				
Grade	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Hemoglobin																1																					1	2	1			1		1			2				
PCV											1					1					1					2											2	2	1			1		1			4				
Thrombocytopenia																																															1				
Thrombocytosis																																																			
Leucopenia	2					1										1					2					1																					2				
Neutropenia																																																		
Monocytosis																																																		
Lymphopenia		1				1					1					1					1	2					2																		1			1	1			
Lymphocytosis																																															1				
Left shift																																																	2		
Eosinophilia	1										1										1															1															
Basophilia						3					1											1																									1				
Lethargy																1														1					1															
Weight loss											1					1																			1										2				
Anorexia												1																																						
Dehydration																																																		
Diarrhea																																																		
Vomiting						1						1				1																																		
Albumin low																																																		
AST																																																		
Calcium low																																																		
Creatinine											1																																							
Apnea																																																		

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Table 3. (*Continued*)

	Post-operative (day 14)																						Follow-up (day 45)																					
	Control					Dose 1					Dose 2					Dose 3					Dose 4					Control					Dose 1					Dose 2					Dose 3					Dose 4				
Grade	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Hemoglobin											3					1																																	
PCV											3					1																																	
Thrombocytopenia																					1										1					1												
Thrombocytosis	1																																														
Leucopenia	1					1										1					1	2				2					1					1					1					1			
Neutropenia					1																1					1																1					
Monocytosis																																															
Lymphopenia		1												1		1	1				1																1					1		1			
Lymphocytosis																																															
Left shift	1																																														
Eosinophilia	2					1					1										1					2					1										2					
Basophilia						1										1										1																1					
Lethargy		1																			1						1																				
Weight loss	2	2				3					2	1				1					3	1				1	1				1					1					2	1				3				
Anorexia		1														1					2		1																							
Dehydration		1																				1																								
Diarrhea																1																														
Vomiting		1														1	1				1	1																								
Albumin low																																															
AST																																															
Calcium low																																															
Creatinine				1							1																																					
Apnea																																															

Control, Control cats ($n = 4$); dose 1, 50 μg feGM-CSF ($n = 4$); dose 2, 250 μg feGM-CSF ($n = 8$); dose 3, 750 μg feGM-CSF ($n = 6$); dose 4, 1250 μg feGM-CSF ($n = 6$). PCV, packed cell volume; AST, aspartate aminotransferase.

Statistical analysis

For all tested parameters documenting an AE, there were no significant differences between the treated groups themselves as well as between treated and control cats.

ELISA

According to the standard curve, plasma feGM-CSF-concentrations between 0.14 μg/ml and 1.0 μg/ml could be measured. Detectable plasma concentrations of feGM-CSF were found in sample sets of four study cats. As these concentrations were already detected in samples from day −14 and had not increased during treatment, they had to be considered as physiological and not as treatment related (data not shown).

ELISAs for feGM-CSF in supernatants from cultured tumors of study cats were positive. However, as in some instances, supernatants of cultured tumors of untreated control cats proved positive for feGM-CSF, the authors were confronted with discriminating between endogenous and treatment-related GM-CSF expression. To demonstrate that the treatment results in expression of the transfected gene, seven cats were treated with the huGM-CSF gene. All seven cats presented positive for huGM-CSF expression and the control experiments with feGM-CSF-positive tumor culture supernatants from feGM-CSF gene vaccinated study cats demonstrated that there was no cross-reactivity of the huGM-CSF ELISA with feGM-CSF (Table 4). Samples from untreated control cats were also negative for huGM-CSF. Hence, these results demonstrate that intratumoral magnetofection of a cytokine gene according to the employed treatment protocol results in expression of the transfected gene.

Preliminary response and recurrence results

Although assessment of the recurrence rate was not the primary aim of the study, the preliminary results are reported here and are shown in Table 2. Passing day 360 follow-up evaluation, ten out of 20 cats treated according to protocol had no evidence for local recurrence, but two of these 20 had developed detectable metastases of the lungs without local recurrence. Eight cats developed a local recurrence, one of them with detectable lung metastases.

Table 4. Results of the huGM-CSF ELISA measured from 24-h supernatants of cultured cat tumors

Cat no.	Treatment on days prior to surgery	Applied gene	huGM-CSF (pg/ml) in 24-h supernatants
I	1	huGM-CSF	8.01
II	1	huGM-CSF	104.7
III	7	huGM-CSF	305.79
IV	7	huGM-CSF	423.12
V	7	huGM-CSF	599.86
VI	14 and 7	huGM-CSF	311.24
VII	14 and 7	huGM-CSF	412.46
VIII	14 and 7	feGM-CSF	0.0
IX	14 and 7	feGM-CSF	0.0
X	Surgery only	None	0.0

huGM-CSF, human granulocyte-macrophage colony-stimulating factor; feGM-CSF, feline granulocyte-macrophage colony-stimulating factor.

Discussion

As shown in various studies in animals and humans, GM-CSF has great anti-tumor potential [23,31]. However, it has also been shown in a murine model that GM-CSF secreting vaccines can exert dose-dependent effects (either imunostimulatory or immunosuppressive) mediated by the induction of myeloid suppressor cells. This inhibitory effect is determined by systemic and not by local secretion of GM-CSF. Myeloid suppressor cells are only observed in conditions in which the systemic levels of GM-CSF exceed a certain threshold and mediate T-cell unresponsiveness [40].

Therefore, magnetofection was used in the present study to ensure local gene transfer and to avoid systemic transfection. This technique is based on the principle of magnetic drug targeting, where gene vectors are associated with magnetic nanoparticles [41], and has been shown to enhance transfection efficiency of viral and nonviral vector systems by up to several hundred-fold [42]. Nonviral gene delivery systems have clear advantages compared to viral vectors in terms of simplicity of use, easiness of large-scale production and lack of specific immune response. Immune responses, the risk of endogenous virus recombination and oncogenic effects are the greatest problems occurring with viral vectors [43]. However, most nonviral vectors display lower transfection efficiency than viral vectors. As shown in previous cell culture studies, the magnetic forces used for magnetofection lead to an accelerated sedimentation of magnetofectins on the cell surface, but do not interfere with the endocytic uptake mechanism [44]. This allows gene therapy with low doses of plasmid, and therefore lower toxicity, but with efficient transfection and gene expression.

The present study aimed to determine possible toxicity and feasibility of gene therapy with plasmid coding for feGM-CSF in cats with fibrosarcomas. In humans, adverse effects of GM-CSF include fever, myalgia, rash, injection site reactions [45], eosinophilia and leucocytosis [46]. In the present study, none of these side-effects were seen. Few mild changes in blood parameters were observed in cats of all dose groups throughout the whole study period. Grade 3 lymphopenia could only be seen in cats of the two highest dose groups, but the lymphopenias in these groups were already detected at the initial check-up and so the correlation grade for these events is unrelated (CG 1) to the conducted treatment. The statistical analysis did not reveal any statistical significant difference for blood parameters within a dose group (day −14 versus day 0) or between dose groups, but this observation must be

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interpreted with caution because of the relatively small number of cats in each group. As the changes in blood parameters observed in cats after systemic application of GM-CSF [47] were not detected in cats from this trial, we can state that systemic levels of GM-CSF that can lead to immunosuppression were not reached.

Death of cat no. 10 (which died 6 days after the first injection of feGM-CSF gene therapy) can be considered as unlikely (CG 2) to be correlated with the treatment. Concerning the findings of the autopsy, it is suggested that the cat died because of a hyperacute course of feline panleukopenia. Additionally, no relevant toxicity occurred in the three cats added to the second dose group. Cat no. 11, which died at induction of anesthesia, had no history of any diseases except for two former fibrosarcoma surgeries in yearly intervals. The owner consented to an autopsy in which restrictive cardiomyopathy with chronic congestion of the liver were the only abnormal findings. For that reason, it is likely that the cat had a decreased anesthesia tolerance so that this adverse event was probably not (CG 2) correlated with gene therapy. This was the only event occurring during anesthesia or surgery.

An underlying cause for the symptoms of vomiting and anorexia in cat no. 13 could not be found but, as the owner reported these symptoms at the initial check-up, the problem is unlikely to be related (CG 2) to the additional therapy. The acute renal failure that cat no. 16 developed 7 days after surgery is clearly unrelated (CG 1) to gene therapy as this cat was in the control group. The reasons for the renal failure in this 4-year-old cat are decreased renal perfusion during anesthesia alone or in combination with the administration of potential nephrotoxicants, such as nonsteroidal anti-inflammatory/analgesic agents. Some cats showed mild lethargy on days 2–14 after surgery but, as the control cats showed the same symptoms, it was attributed as being unrelated (CG 1) to gene therapy. As shown in Table 3, the majority of cats had decreased hemoglobin and PCV levels on days 2 and 14 due to the blood loss during surgery.

In dose group 4, three out of four cats showed lethargy, anorexia or vomitus on day −7 or 0. For further evaluation of these AEs, two additional cats were enrolled and treated with the highest dose of plasmid coding for feGM-CSF. As these two cats showed no signs of toxicity during and after the two injections, this highest dose (1250 μg) applied by magnetofection in cats with fibrosarcomas was found to be both feasible and tolerable. This dose of 1250 μg plasmid coding for feGM-CSF was determined to be the highest given dose in the present study because further escalation would have demanded an increase of injection volume to 1000 μl and thus a change in study settings. To escalate only the plasmid dose, and therefore change the 1 : 1 ratio of plasmid DNA and magnetic iron oxide nanoparticles, is not possible because of precipitation.

To evaluate possible elevated systemic levels of feGM-CSF as a result of local gene therapy with the feGM-CSF gene, an ELISA was performed. No systemic levels of feGM-CSF developing during the treatment period could be detected. As measurable levels were detected at the initial check-up in four cats and in the supernatants used as positive controls, the sensitivity of the conducted ELISA was satisfying. As stated by the manufacturer, there is a high specificity for the used ELISA as there is no cross-reactivity or interference with recombinant hGM-CSF, murine, porcine and rat GM-CSF. Thus, it can be stated that the local transfection of the feGM-CSF gene by magnetofection as carried out in the present study does not lead to detectable elevated systemic levels of the cytokine.

The ELISA for systemic levels of feGM-CSF in plasma does not reflect the actual cytokine profile in the milieu of the tumor cells. For this reason, an ELISA of the supernatants of cultured tumor cells from treated and control cats was performed. As some samples from control cats were positive and some were negative for feGM-CSF expression, it was impossible to relate positive feGM-CSF ELISA measurements unequivocally to the expression of the transfected gene. To obtain supportive evidence, seven cats were injected with the huGM-CSF gene. As the persistence of transfected gene expression in the tumors has been unknown so far, cats were subjected to different treatment schedules, albeit only with a very limited number of animals per group. A total of five cats received two injections as in the feGM-CSF study group; however, in three of these animals, the tumor was removed 1 day after the second injection. Two other animals received only one treatment with the human GM-CSF gene and the tumor was excised 1 day after the treatment. All samples proved positive for huGM-CSF whereas controls were negative, generating convincing evidence that the transfected cytokine gene is expressed. Interestingly, samples from double-dosed cats displayed higher expression levels than the samples from single-dosed cats, even when the time span between the last dosing and surgical removal of the tumor was 7 days. This indicates that the persistence of transfected gene expression can be sufficient to stimulate a putative immune response during the 2-week 'incubation' period prior surgical removal of the tumor.

Clearly, these findings are only a first step towards a more complete monitoring of the expression of the transfected therapeutic gene and its consequences. Ongoing work focuses on using polymerase chain reaction methods to discriminate between endogenous and exogenous feGM-CSF and on characterizing putative anti-tumor immune responses. Techniques such as enzyme-linked immunospot (ELISPOT) or the detection of intracellular cytokines by multiparameter flow cytometry should be employed. A correlation of measured immunological parameters with clinical outcome (in humans) has already been demonstrated, but this correlation was not found in all settings [48]. However, applying such techniques in the feline system is quite a challenge due to the limited availability, or even absence, of feline-specific markers and antibodies.

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Although this was a phase I trial, and was not designed to test any clinical benefit, the observed recurrence rates were nonetheless encouraging. Clearly, it is not possible to relate the observed recurrence rates with statistical significance to the different dosage groups because of the small number of patients in each group. It will be left to a subsequent phase II trial to include the monitoring of immune responses with a high enough patient number to allow statistically significant results.

This phase I clinical trial revealed feGM-CSF gene delivery by magnetofection to be a well tolerated, feasible and promising neoadjuvant treatment in cats with fibrosarcomas. In conclusion, a dose of 1250 µg feGM-CSF plasmid DNA was identified for phase II testing. This dose appears to be safe and feasible and may be associated with the induction of an anti-tumor immune response.

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