Research in Veterinary Science 97 (2014) 386–390

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Murine xenograft model demonstrates significant radio-sensitising effect of liposomal doxorubicin in a combination therapy for Feline Injection Site Sarcoma

H. Petznek [a,*], M. Kleiter [b], A. Tichy [c], A. Fuchs-Baumgartinger [d], C. Hohenadl [a]

[a] Institute of Virology, Department for Pathobiology, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria
[b] Radiooncology and Nuclear Medicine Platform, Department for Companion Animals and Horses, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria
[c] Bioinformatics and Biostatistics Platform, Department for Biomedical Sciences, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria
[d] Institute of Pathology and Forensic Veterinary Medicine, Department for Pathobiology, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria

ARTICLE INFO

Article history:
Received 25 December 2013
Accepted 9 July 2014

Keywords:
FISS
Doxorubicin
Radiation
Primary feline sarcoma cell culture
Xenograft tumour mouse model

ABSTRACT

Therapy of cats suffering from feline injection site sarcomas (FISS) is still a challenging problem, as the recurrence rate after surgery is up to 70%. Four FISS-derived primary tumour cell lines and corresponding xenograft tumour mouse models were established to evaluate the efficacy of a concomitant chemo-/radiation therapy with doxorubicin. *In vitro*, strongly depending upon the timing of administration, pre-treatment with 0.25 μmol doxorubicin resulted in a significant enhancement of radiation-induced (3.5 Gy) tumour cell death. This result was confirmed *in vivo*, where only the combined chemo-/radiation therapy resulted in a significant reduction in tumour growth compared to the respective mono-therapies with either doxorubicin or radiation. These results support the use of the concomitant chemo-/radiation therapy for adjuvant treatment of FISS, particularly in advanced or recurrent disease where surgery alone is no longer feasible.

1. Introduction

The development of feline injection site sarcomas (FISS) following vaccination or injection of therapeutic agents has been extensively documented for more than 20 years (Munday et al., 2011; reviewed in Martano et al., 2011). In the genetic background of mutations or deregulated expression of oncogenes and tumour suppressor genes, the permanent stimulation through cytokines and growth factors at sites of chronic inflammation leads to transformation of fibroblasts and myofibroblasts into neoplastic cells (Hendrick and Brooks, 1994; reviewed in Martano et al., 2011).

The treatment of choice for FISS is surgery (Dillon et al., 2005), although recurrence rates after excisions vary between 30 and 70% (Banerji and Kanjilal, 2006; Martano et al., 2011; Seguin, 2002), and tumours with clean surgical margins have shown a recurrence rate of 19% (Giudice et al., 2010). This might indicate that some recurrences are actually new neoplastic transformations of activated cells within the wound-healing tissue, since after surgery cytokines and growth factors are again released by inflammatory cells recruited to the site of intervention, thereby exerting a tumour-promoting effect on proliferating fibroblasts (Giudice et al., 2010; Martano et al., 2005). This raises the question of how radical surgical resections should be and if a more conservative approach would not be as promising, particularly when combined with other therapies (Cohen et al., 2001). Additional treatment options include chemotherapy, radiation- and immunotherapy, or combinations of these. A promising combination to be evaluated in this context is the administration of doxorubicin and radiation. Aside from killing tumour cells directly through the inhibition of topoisomerase II and free oxygen radical formation, doxorubicin can enhance the effect of radiation by accumulating unwound DNA in cells, which consequently are sensitised to radiation. This enhancement has been shown in human cancer cell lines (Supiot et al., 2005), murine xenograft models (Charrois and Allen, 2003; Harrington et al., 2000) and humans (Koukourakis et al., 2000). Such a combined therapy with doxorubicin and radiation has also been reported for the treatment of FISS in cats, but with inconsistent and often disappointing results. This may be mainly due to the applied treatment regimen. In several studies doxorubicin was either administered directly before radiation or weeks after the last radiation cycle (Bregazzi et al., 2001; Hahn et al., 2007), thereby missing the radio-sensitising effect of doxorubicin.

In the present study, *in vitro* and *in vivo* FISS model systems were established to investigate the effect of doxorubicin in combination

* Corresponding author. Tel.: +43 1 25077-2331; fax: +43 1 25077-2390.
E-mail address: Helga.Petznek@vetmeduni.ac.at (H. Petznek).

http://dx.doi.org/10.1016/j.rvsc.2014.07.008
0034-5288/

with concomitant radiation in a standardised setting. Various treatment regimens were first tested using primary, well characterised FISS-derived cell lines. A corresponding xenograft tumour mouse model was subsequently established with these cell lines, allowing evaluation of the combination therapy in a randomised study with a statistically substantiated outcome.

2. Materials and methods

2.1. Cell lines

Cell line FFS1 was derived from tumour specimens of a FISS of a 6–year-old male European Shorthair cat; FFS2 and FFS2ST cells originated from a FISS of a 9-year-old female European Shorthair cat. Tumour tissue was digested for 2 h at 37 °C in phosphate-buffered saline (PBS, PAA Laboratories) containing 2 mg/ml collagenase (Worthington). The resulting cell suspension was centrifuged at 180 × *g* and re-suspended in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% foetal bovine serum (FBS, PAA Laboratories) to be seeded in 75 cm^2 cell culture flasks (Sarstedt) and incubated in a humidified atmosphere at 37 °C and 5% CO_2. For passaging, cells were washed twice with PBS and then exposed to Trypsin-EDTA (0.05%, PAA Laboratories) for 3 min. The reaction was stopped by adding DMEM/10% FBS and cells were transferred into a new flask. During the first two passages, FFS2 cells that did not dissolve from the surface within 3 min were separated and renamed FFS2ST. A fourth cell line (FFS1rec; see Supplementary Fig. S4) was derived from a murine FFS1 xenograft tumour after 40 *in vivo* passages. For re-cultivation, the excised tumour tissue was treated as described above. The feline origin of the FFS1rec cells was demonstrated by quantitative staining with a mouse monoclonal antibody specific for feline MHC class I (CF298A, VMRI). Before cells were used in the respective experiments, they were adapted to growth in DMEM/5% FBS.

2.2. Growth kinetics

To evaluate growth kinetics of the four cell lines, 5×10^4 cells were seeded per well of a six-well plate and cultivated in DMEM/5% FCS. Triplicate samples were trypsinised and the number of cells determined by automated cell counting (CasyTT, Schärfe Systems GmbH) on Days 2, 4, 7 and 9.

2.3. In vitro evaluation of a doxorubicin/radiation combination therapy

Two days before treatment started, 5×10^4 cells were seeded per well of a six-well plate and cultivated in DMEM/5% FBS in a humidified atmosphere at 37 °C and 5% CO_2. For treatment, cells were exposed to 0.25 µmol doxorubicin (doxorubicin-hydrochloride; Adriblastin, Pfizer) and/or irradiated with a single dose of 3.5 Gy (linear accelerator Siemens Primus 6 MV, Siemens). Cells were covered with doxorubicin containing medium for 8 h; after removal, cells were washed twice with PBS and fresh DMEM/5% FBS was added. Radiation (Rad) was either performed in the presence of doxorubicin 4 h after addition of the drug to the medium (D0) or 24 h later (D1), i.e. after removal of doxorubicin (Table 1). Besides washing and medium change, control cells were kept untreated. On Days 2, 5, and 7 the number of surviving cells was determined by automated cell counting (CasyTT, Schärfe Systems GmbH). Samples were analysed in triplicates.

2.4. Tumour xenograft mouse model

FFS1, FFS2 and FFS2ST cells were subcutaneously (SC) injected into immunodeficient Hsd:Athymic Nude-*Foxn1*^{nu} mice (n = 8) to investigate their tumourigenic potential. Animals were anaesthetised by intraperitoneal (IP) injection of 10 mg Ketamine/0.4 mg Xylazin/100 g body weight (BW). The left side of the thorax was injected with 5×10^6 cells re-suspended in 100 µl PBS. The resulting tumours were measured twice a week with a caliper and tumour volume was calculated according to the formula length × width × width/2. For passaging of FFS1 tumours, mice were euthanised when tumours had reached a volume of approximately 1000 mm^3. The tumour was excised and a piece of approximately 1.5 mm^3 was implanted into the next mouse. For this purpose, mice were anaesthetised as described above; after a short incision into the skin a small cavity was prepared with forceps and the tumour piece was placed into it. The wound was closed with one or two suture clips. Implantation was performed either at the left side of the thorax (during passaging) or the lateral part of the left thigh (treatment evaluation).

Table 1
In vitro treatment regimen.

Group	Doxorubicin (Dox)	Radiation (Rad)	
		D0	D1
1	0.25 µmol	No	No
2	No	3.5 Gy	No
3	No	No	3.5 Gy
4	0.25 µmol	3.5 Gy	No
5	0.25 µmol	No	3.5 Gy
6	No	No	No

2.5. Histological analysis

Parts of the original FISS tumours and FISS tumours grown in mice, respectively, were fixed in 4% neutral buffered formalin, embedded in paraffin. Two to three micrometre sections were stained with haematoxylin and eosin (HE). The tumours were graded according to the scheme of Couto et al. (2002).

2.6. Doxorubicin administration in mice

For *in vivo* studies liposomal doxorubicin was used, since it has been shown previously to be more effective in enhancing radiotherapy (Harrington et al., 2000).

Pegylated liposomal doxorubicin (Caelyx, Janssen Cilag GmbH) was diluted 1:10 with glucose solution (5%, Fresenius) to a final concentration of 0.2 mg/ml. For injection into the lateral tail vein (IV), mice were anaesthetised as described and put onto a warming plate (37 °C) for 5 min.

2.7. In vivo evaluation of the combination treatment

For testing the efficacy of the combination treatment with doxorubicin and radiation, pieces of FFS1 tumours were implanted into the lateral part of the left thigh of Hsd:Athymic Nude-*Foxn1*^{nu} mice (n = 42) as described above. Tumour size was measured twice a week and as soon as it exceeded 100 mm^3, mice were randomly divided into six different treatment groups. The first group received 3 mg/kg Caelyx, the second and third groups were irradiated twice with 3.5 Gy and 5 Gy, respectively, at a time interval of 24 h; Groups 4 and 5 received the combination treatment of 3 mg/kg Caelyx and two times radiation with either 3.5 Gy or 5 Gy. Radiation was performed 24 h and 48 h after Caelyx injection. The last group was mock-treated receiving 5% glucose solution. For health monitoring, mice were inspected daily and weighed twice a week. Mice were euthanised as soon as tumour size exceeded 1500 mm^3 or tumours ulcerated. Remaining mice were sacrificed on Day 182 after beginning of treatment.