The influence of fractionated radiation therapy on plasma vascular endothelial growth factor (VEGF) concentration in dogs with spontaneous tumors and its impact on outcome

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Abstract

BACKGROUND AND PURPOSE: Vascular endothelial growth factor (VEGF), a specific pro-angiogenic factor is proposed to be involved in cancer progression and resistance to radiation therapy by promoting angiogenesis and by protecting endothelial cells from radiation induced apoptosis. The aim of this study, was first to assess the influence of ionizing radiation on plasma VEGF concentration in spontaneous canine tumors during fractionated radiation therapy with curative or palliative intent and second to analyze plasma VEGF concentration as predictor for treatment outcome.

PATIENTS AND METHODS: For plasma VEGF analysis a human VEGF enzyme linked immunosorbent assay was used. Sixty dogs with various tumor types were included in this study. Dogs were irradiated with either low dose per fx (3-3.5 Gy per fraction, total dose: 42-49 Gy, group A: curative intent) or high dose per fx (6-8 Gy per fraction, total dose: 24-30 Gy, group B: palliative intent). Blood samples were taken before and after dose application at certain time points during therapy. Follow-up evaluation was performed for analysis of time to treatment failure and survival.

RESULTS: Repeated measures analysis showed no increase of plasma VEGF in dogs treated with fractionated radiation therapy (group A and B). Dichotomizing baseline plasma VEGF into two groups with high and low plasma VEGF, resulted in shorter time to treatment failure in dogs with high plasma VEGF levels (TTF, group A: P=0.038, group B: P=0.041). CONCLUSIONS: This study demonstrated that dogs with a plasma VEGF level higher than 5 pg/ml had a poorer outcome after radiation therapy. It is therefore, suggested, to use plasma VEGF as predictor for treatment outcome in radiation therapy.
Clinical radiobiology

The influence of fractionated radiation therapy on plasma vascular endothelial growth factor (VEGF) concentration in dogs with spontaneous tumors and its impact on outcome

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Abstract

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Conclusions: This study demonstrated that dogs with a plasma VEGF level higher than 5 pg/ml had a poorer outcome after radiation therapy. It is therefore, suggested, to use plasma VEGF as predictor for treatment outcome in radiation therapy.

Keywords: Canine; Plasma VEGF; Time to treatment failure; Survival; Radiation

Tumor angiogenesis is an important factor in disease progression and formation of metastasis [12]. One major pro-angiogenic factor is vascular endothelial growth factor (VEGF). VEGF promotes tumor angiogenesis, endothelial cell survival and vessel maintenance of immature vessels [12,13]. VEGF is involved in development and growth of a wide variety of different tumors [15,17]. VEGF is actively secreted from tumor cells and its soluble form (VEGF\(_{165}\)) is detectable in the blood compartment [9].

Exposure of tumor cells to ionizing radiation can increase VEGF mRNA [2] and protein concentration [14]. Ionizing radiation can lead to phosphorylation of the PI-3kinase via the epidermal growth factor receptor (EGFR) in tumor cells. This is the first step in activating the ERK pathway, a mitogen activated protein kinase (MAPK) pathway. This pathway is mediating cell survival and upregulation of VEGF expression in tumor cells [4,9]. In human squamous carcinoma cells the activation was dependent on the radiation dose, with low doses (1 Gy) causing prolonged activation of the ERK pathway and higher doses (6 Gy) having a weaker activation [4].

Cells incubated under hypoxic conditions had increased VEGF expression [22,25]. This increase was mediated by the hypoxia inducible factor-1 (HIF-1). HIF-1 consists of two subunits, HIF-1\( \alpha \) and HIF-1\( \beta \). Under normoxic conditions HIF-1\( \alpha \) is rapidly degraded [22,25], but in hypoxic cells the heterodimer HIF-1 can bind to DNA at specific regions, called hypoxia responsive elements (HRES) which includes a 28-bp element that is sufficient to mediate upregulation of VEGF transcription [19]. Several studies have associated HIF-1 expression with human cancer progression, such as head and neck cancer, ovarian cancer and esophageal cancer [11,33].
Recently, an association between tumor hypoxia and elevated systemic levels of VEGF in head and neck cancer has been shown [3]. In dogs with spontaneous tumors low hemoglobin levels correlated with high plasma VEGF levels [27]. In this study, a significant difference of plasma VEGF concentration in different tumor types was found with high levels in carcinoma, osteosarcoma, and melanoma [27].

Low pre-operative serum VEGF levels (<575 pg/ml) were shown to correlate with increased disease free survival in human patients with colorectal cancer indicating that serum VEGF was a good predictor for outcome [29]. When serum and plasma VEGF in patients with primary colorectal carcinoma were analyzed separately, both, high serum and high plasma VEGF concentrations predicted for shorter survival, but significance was only reached for serum VEGF values [29]. In contrast, high plasma VEGF was shown to be a significant predictor of reduced overall survival and of earlier onset of local recurrence for patients with breast cancer [23,30].

Therefore, we were interested whether (1) the influence of a low dose per fx (3–3.5 Gy per fraction, total dose: 42–49 Gy, group A: curative intent) or high dose per fx (6–8 Gy per fraction, total dose: 24–30 Gy, group B: palliative intent) on the VEGF release from tumor cells can be measured in plasma of dogs with spontaneous tumors, and (2) if baseline plasma VEGF as well as the course of plasma VEGF during therapy can predict time to treatment failure (TTF) and survival.

Material and methods

Patient selection

Sixty tumor bearing dogs were included in this study. There were 38 male dogs and 22 female dogs (12 neutered male and nine neutered female dogs). The median age of patients was 9.0 years (range 3-16 years). The study group included 35 pure breed dogs of various breeds and 25 mixed breed dogs. The median weight of these dogs was 29.7 kg (range 5.1-66 kg). The routine work up included in all animals a complete physical exam, blood count, serum chemistry, thoracic radiographs, biopsies of the primary tumor using a needle punch biopsy (Tru-cut, Baxter General Health Care, Deerfield, Illinois) or an incisional biopsy for routine histopathological diagnosis. Fine needle aspiration of enlarged lymph nodes was performed using a 22-gauge needle and a 5 ml syringe. Further diagnostic work up was done as indicated. Patients with sarcomas of soft tissue (n=28) or bone origin (n=14), carcinomas (n=9), oral melanomas (n=6) and three dogs with acanthomatous epulids were included (Table 1). All patients were staged based on the world health organization (WHO) system [24] (Table 1). For 17 patients no staging system was applicable. The length, width and depth of tumors were measured and the volume was calculated using the rotation ellipsoid formula ($abc/6$). The median tumor volume was 35.7 cm$^3$ (range 0.1-392.5 cm$^3$). The initial tumor response at the end of fractionated radiation therapy was defined as no response (unchanged tumor volume), partial response (decreased tumor volume), or complete response (no macroscopic tumor disease) (Table 1). Acute side effects of normal tissue were recorded by using the toxicity criteria suggested by the VRTOG [18]. Informed consent of owners was obtained.

Anesthesia protocol

All dogs were anesthetized for radiation therapy. Midazolam (Dormicum®, Roche Pharma AG, Reinach, Switzerland) was injected at a dosage of 0.125 mg/kg intravenously immediately followed by propofol (Propofol®, Fresenius Kabi AG, Stans, Switzerland), slowly administered to effect. Anesthesia was maintained after intubation with isoflurane and oxygen (Forene®, Abbott AG, Baar, Switzerland). During anesthesia dogs were monitored with pulse oxymetry and ECG.

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<th>Tumor related parameters and treatment response</th>
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<td>Tumor histology</td>
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a No response to therapy.
b Partial response.
c Complete response.
Treatment regimens

All dogs were treated with radiation therapy. The treatment was chosen based on tumor volume, tumor stage, tumor histology and overall health of the dog. To be included all dogs had to have a measurable tumor mass. Radiation therapy was given with a linear accelerator (BBC Dinary 20) using 6 MV photons or 5- to 16-MeV electrons. If indicated, computer treatment planning was performed with CadPlan 6.0. A total of 21 dogs (group A) were irradiated with a low dose per fraction (fx) with a total dose of 42-49 Gy (curative intent). Dogs were treated either with 3.5 Gy fractions on a Monday, Tuesday, Thursday and Friday schedule or a daily fractionation scheme with 3 Gy fractions was used. Thirty-nine dogs (group B) were treated with a high dose per fx with a total dose of 30 Gy delivered in five fractions, or a total dose of 24 Gy delivered in three or four fractions (palliative intent). Detailed data are given in Table 2.

Blood samples and VEGF assay

Three milliliters of blood were collected into sterile CTAD tubes (Beckton and Dickinson Vacutainer System, France) and placed on ice. CTAD tubes contained sodium citrate, theophyllin, adenosine and dipyridamine allowing maximal platelet stabilization. The tubes were centrifuged within 15 min at 2500 × g for 30 min at 4 °C [31]. The resulting plasma was separated and stored immediately at −80 °C. The technique of blood sampling and handling of probes has been published previously [10,31]. Thrombocyte count in CTAD plasma was measured and the amount was at the detection limit (<5000). A blood sample, taken before therapy, served as baseline plasma VEGF. In group A, blood samples were always taken before the 1st and after the 3rd, 6th, 8th, 10th, 12th and 14th fraction. Blood samples were obtained before the 1st and after every following fraction in group B.

VEGF concentration was determined by using the Human VEGF enzyme linked immunosorbent assay (ELISA, R&D System, Inc., Abingdon, United Kingdom) designed for detection of VEGF165. This ELISA has already been proven reliable for plasma VEGF evaluations in dogs in recent studies [6,7,27]. The VEGF ELISA was accomplished using the protocol from the manufacturer. The standard curve was adapted for low concentrations and the detection limit was tested up to 1 pg/ml. A standard curve was assayed for each microtiter plate. After thawing, every aliquot was assayed twice and the mean value was taken for statistical analysis. Inter- and intra-donor variations have been tested in healthy dogs [28].

Outcome measures

Time to treatment failure (TTF) was defined to be the interval from the date of completion of radiation therapy to the date of disease recurrence or progression and/or the observation of metastasis. The survival time was defined as the time interval from start of therapy to death/euthanasia. Follow-up evaluation was performed in our clinic or by the referring veterinarian 3 and 6 months after completion of therapy and then every year thereafter.

Statistical analysis

Plasma VEGF levels showed a skewed distribution, therefore plasma VEGF levels were transformed logarithmically before analysis (plasma ln VEGF). Statistical analysis (StatView Version 4.0 statistical software application, Abacus concept) was performed using the descriptive statistics and for correlation of plasma VEGF and tumor response the Kruskal-Wallis test was used. Estimates of probabilities for time to treatment failure (TTF) were calculated by the Kaplan-Meier method, and differences between groups were tested with the log rank statistic. The plasma VEGF concentration was scored as low if plasma VEGF was less than the 50th percentile of baseline plasma VEGF of all tumor bearing dogs. The median plasma VEGF for all dogs was 5.2 pg/ml. Therefore, groups were dichotomized in patients having a plasma VEGF concentration lower or higher 5 pg/ml. To analyze the effect of ionizing radiation on VEGF release over time the repeated measures analysis with Greenhouse-Geisser correction was used (SPSS program). This test was distinguished from MANOVA to test within subject effects. For this analysis plasma VEGF levels after dose application were subtracted from baseline plasma VEGF. P values ≤0.05 were considered significant.

Results

Baseline analysis

Dogs receiving a low dose per fx, high total dose (group A)

Median plasma VEGF was 6.2 pg/ml. Dogs having no response (NR) to radiation therapy had median plasma VEGF of 10.1 pg/ml, dogs with a partial response (PR) had median plasma VEGF of 7.5 pg/ml and dogs with a complete response (CR) had median plasma VEGF of 5.7 pg/ml. But differences between groups were not statistically significant (P = 0.67).

Dogs receiving a high dose per fx, low total dose (group B)

Median plasma VEGF in this group was 4.8 pg/ml. For non-responders the median plasma VEGF was 9.7 pg/ml, dogs

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<td>Fractionation scheme for curative (group A) and palliative (group B) treatment</td>
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<sup>a</sup> Dogs receiving a low dose per fx, with a high total dose.

<sup>b</sup> Dogs receiving a high dose per fx, with low total dose.
Plasma VEGF measured over the course of radiation therapy

**Dogs receiving a low dose per fx, high total dose (group A)**

Change of plasma VEGF during radiation therapy was not statistically different ($P=0.7$). Plasma VEGF during radiation therapy in dogs with sarcomas was stable (sarcoma: $P=0.57$). The mean plasma VEGF level of dogs with carcinoma increased slightly over the course of radiation therapy although this was not significant ($P=0.3$). Plasma VEGF levels directly before and after dose application did not differ. The repeated measures analysis with the covariables: tumor volume, tumor stage and radiation reaction were computed separately and none of the covariables influenced plasma VEGF during therapy (tumor volume: $P=0.2$, tumor stage: $P=0.3$, radiation reaction: $P=0.4$).

**Dogs receiving a high dose per fx, low total dose (group B)**

Plasma VEGF in dogs treated with a high dose per fraction remained unchanged ($P=0.8$). The same was seen when patients were grouped according to tumor histology, such as melanoma ($P=0.6$), sarcoma ($P=0.5$) and carcinoma ($P=0.8$). Plasma VEGF levels directly before and after dose application did not differ. None of the covariables influenced the course of plasma VEGF (tumor volume: $P=0.7$, tumor stage: $P=0.4$, radiation reaction: $P=0.5$) (Fig. 1).

**Treatment outcome**

Comparison of dogs irradiated with a curative intent (low dose per fx, high total dose) to dogs irradiated with a palliative intent (high dose per fx, low total dose) resulted in significantly longer survival in dogs irradiated with a low dose per fraction (high total dose) (data not shown).

Therefore, the survival analysis was computed for both groups separately. Dogs remaining alive at last follow-up or dogs that died of unrelated reasons were censored.

**Dogs receiving a low dose per fx, high total dose (group A)**

The median follow-up time was 524 days (mean: 490 days). At the time of analysis 14 out of 21 dogs had died because of their cancer disease. Seven dogs were censored, five dogs were still alive at the time of analysis and two dogs died of unrelated reasons. The median TTF of the 14 dogs was 153 days (31–440 days). When baseline plasma VEGF was dichotomized into two groups with plasma VEGF levels of $\leq 5$ pg/ml ($n=7$), respectively $>5$ pg/ml ($n=7$), then dogs with a plasma VEGF level higher 5 pg/ml had a significantly shorter TTF ($P=0.04$) (Fig. 2). The median TTF of dogs with a plasma VEGF $\leq 5$ pg/ml was 243 days and for dogs with plasma VEGF $>5$ pg/ml the median TTF was 136 days. This difference in pretreatment plasma VEGF also resulted in a shorter survival time ($P=0.04$).

Further, we looked at the course of plasma VEGF during radiation therapy and its influence on TTF and survival. Dogs were separated into two groups. One group had increasing or decreasing plasma VEGF levels. However, TTF and survival were not influenced by increasing or decreasing plasma VEGF levels.

**Dogs receiving a high dose per fx, low total dose (group B)**

The median follow-up time was 93 days (mean: 125 days). Out of 39 dogs, 10 dogs were censored, of which three were still alive at the time of analysis, five dogs died of unrelated causes and two dogs were lost to follow-up. Dogs with high plasma VEGF ($>5$ pg/ml) had a significantly shorter TTF ($P=0.04$) (Fig. 2). The median TTF for dogs with plasma VEGF $\leq 5$ pg/ml ($n=14$) was 102 days and dogs with $>5$ pg/ml...
(n = 15) plasma VEGF had a median TTF of 80 days. This difference in pretreatment plasma VEGF also resulted in a shorter survival time (P = 0.03). The course of plasma VEGF, as mentioned above, was not influencing TTF or survival. Dogs that responded to therapy and had a plasma VEGF level ≤ 5 pg/ml had a significantly lower TTF (P = 0.004) and longer life span (P = 0.005).

Discussion

VEGF is known to protect endothelial cells for radiation induced apoptosis [21, 26, 32]. Therefore, we were interested in changes of plasma VEGF during fractionated radiation therapy with either high or low dose per fraction. However, our study did not reveal a significant increase of plasma VEGF during fractionated radiation therapy in both treatment groups. In vitro, a 2.5-fold increased VEGF concentration has been measured in lung squamous carcinoma cells exposed to a single fraction of 15 Gy [2]. This increase was measurable up to 24 h and it was dependent on the activation of the ERK cascade through activation of the EGFR [2, 9]. The activation of the ERK pathway was dose dependent with increased activity after exposure to a low radiation dose (1 Gy) [4]. Interestingly, we found that dogs with carcinoma irradiated with a low dose per fraction had a slight increase of plasma VEGF, although this was not significant.

The regulatory mechanism of VEGF expression is not only dependent on the activation of ERK. Hypoxia is also a strong stimulus for VEGF expression in tumor cells. Recently, serial oxygen partial pressure measurements (pO2) were done in spontaneous canine tumors during fractionated radiation therapy and the data suggest that tumors may behave differently with increasing or decreasing tumor oxygen levels during radiation therapy [1]. These findings may explain increasing or decreasing plasma VEGF concentrations during therapy in individual dog patients in this study. Additionally, micro vessel density (MVD) seems to negatively correlate with VEGF and positively correlate with tumor oxygenation status [8]. We were able to analyse MVD in seven patients and we found a median MVD of 3.2 vessels per visual field. Dogs with a high plasma VEGF (> 5 pg/ml, n = 2) had a median MVD of 2.2 and dogs with a low plasma VEGF (≤ 5 pg/ml, n = 5) had a median MVD of 4.8. This preliminary result might indicate that tumors with a high MVD are better oxygenated resulting in a lower plasma VEGF. Due to small sample size a definitive conclusion cannot be drawn.

In a previous study [28], we analyzed the course of VEGF during fractionated radiation therapy in a smaller study group and there was a tendency for increased plasma VEGF level in curatively treated dogs. That this tendency did not reach significance in this larger patient group might be due to several factors. First, a counterbalancing effect might occur between ERK pathway activation and changes in the oxygenation status of the tumor. Second, the existence of mechanisms avoiding VEGF release from tumor tissue have been demonstrated. Possibly, binding of VEGF to its receptor and to extracellular matrix occurs and would explain the unchanged plasma VEGF levels. For example, after breast cancer surgery higher VEGF levels were found in wound fluid than in the blood compartment [16]. The lack of significant differences in plasma VEGF levels during radiation therapy might be the simple fact that changes were too small to be detected.

This study demonstrated that high pre-irradiation plasma VEGF levels in dogs with spontaneous tumors resulted in shortened survival times. This was seen in both treatment groups. Interestingly, this prediction was not dependent on tumor histology or tumor stage. Additionally, we found lowest median plasma VEGF levels in dogs with a complete response to therapy in both treatment groups. Correspondingly, palliatively treated dogs (group B) with a low plasma VEGF level that responded to therapy had also a significantly longer TTF and a longer live span. In a human colorectal cancer study, it was also shown that high plasma and serum VEGF concentration prior to surgery predicted decreased overall survival [5, 29].

In a mouse model study, it has been demonstrated that even a minimal increase in plasma VEGF can cause growth of micrometastasis by tipping the balance towards angiogenesis [20]. Therefore, we wanted to analyze if increasing concentrations of plasma VEGF over the course of radiation therapy in individual patients would result in shortened TTF and survival. However, increasing plasma VEGF over time had no effect on survival or TTF.

In conclusion, plasma VEGF appeared to be a good prognostic indicator for dog patients receiving radiation therapy. First, dogs with a low plasma VEGF level are more likely to respond to radiation therapy and second these dogs also have a longer TTF and survival.

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