

Serum and plasma vascular endothelial growth factor in healthy dogs

Fator de crescimento do endotélio vascular no soro e plasma de cães saudáveis

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Abstract

Vascular endothelial growth factor (VEGF) is an angiogenic factor with a key role in physiological and pathological process. It can be measured in several organic fluids, including serum and plasma samples. The aim of this work was to investigate the concentration of serum and plasma VEGF of healthy dogs in order to recommend optimal handling of biological samples for accurate measurement of VEGF. Blood samples of thirty dogs were collected into sterile EDTA tube for plasma analysis and into clot activator tubes for serum analysis. The tubes were centrifuged within 90 minutes of collection at 1400 xg for 10 minutes. VEGF concentration was determined using the quantitative method (ELISA). Serum VEGF level was 26.5 + 13.3pg/mL and plasma VEGF was 11.7 + 16.4 pg/mL ($p = 0.0003$). There was a positive correlation between serum VEGF and platelets ($r = 0.37$, $p = 0.03$) and a negative correlation between serum VEGF and hemoglobin ($r = -0.38$, $p = 0.03$) and between plasma VEGF and hemoglobin ($r = -0.34$, $p = 0.06$). When compared with serum samples it was concluded that plasma samples could be used as an optimal fluid for measuring VEGF in dogs.

Keywords: Angiogenesis. Growth factor. Organic fluids.

Resumo

O fator de crescimento do endotélio vascular (VEGF) é um fator angiogênico com papel importante em processos patológicos e fisiológicos. O VEGF pode ser quantificado em diversos fluidos orgânicos, incluindo amostras de soro e plasma. O presente trabalho investigou a concentração do VEGF no soro e no plasma de cães saudáveis a fim de recomendar o manejo ótimo de amostras biológicas para a determinação dos níveis do VEGF. Amostras de sangue de 30 cães saudáveis foram coletadas em tubos estéreis contendo EDTA para análise do plasma e em tubos com ativador de coagulação para análise do soro. Os tubos foram centrifugados após 90 minutos da coleta a 1.400 x g por 10 minutos. A concentração do VEGF foi determinada com o método quantitativo de ELISA. O nível sérico médio de VEGF foi de 26,5+13,3pg/mL e o plasmático foi 11,7 + 16.4 pg/mL ($p = 0,0003$). Houve correlação positiva entre o VEGF do soro com as plaquetas ($r = 0,37$, $p = 0,03$) e correlação negativa entre o VEGF do soro com hemoglobina ($r = -0,38$, $p = 0,03$) e entre VEGF do plasma com hemoglobina ($r = -0,34$, $p = 0,06$). A comparação dos resultados obtidos nos exames de plasma e soro indicou que amostras de plasma podem ser utilizadas como ótimo fluido para quantificação do VEGF de cães

Palavras-chave: Angiogênese. Fator de crescimento. Fluidos orgânicos.

Introduction

Vascular endothelial growth factor is a multifunctional cellular factor which can induce vascular generation, elevate capillary permeability by directly affecting endothelial cells, stimulate mitosis of endothelium and play a critical role in the formation of a new vascular system and malignant neoplasm development (BREKKN; THORPE, 2001).

VEGF is considered one of the major modulators of angiogenesis in a variety of physiological process,

like wound healing and embryonic development, and plays a role in the physiopathogenesis of diseases like diabetic retinopathy, psoriasis and tumor growth

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Received: 28/08/2013

Approved: 30/10/2013

(FRANK et al., 1995; CARMELIET et al., 1996; FERRARA, 1999).

Vascular endothelial growth factor represents one of the most potent agents promoting tumor angiogenesis. VEGF has angiogenic, mitogenic and vascular permeability enhancing activity specific for endothelial cells, resulting in sprouting of vessels from pre-existing microvessels (VEIKKOLA et al., 2000). VEGF is involved in development and growth of a wide variety of different tumors (HYODO et al., 1998; HEFLER et al., 1999). And plays a role in the formation of distant metastasis (MAEDA et al., 1996). In several types of human cancer the malignant cells produce and release soluble VEGF (KOLCH et al., 1995).

VEGF has been measured in serum and plasma of tumor patients. VEGF is actively secreted from tumor tissue and its soluble form (VEGF165) is detectable in the blood compartment (WARTIOVAARA et al., 1998; SALGADO et al., 1999). VEGF can be released into blood and body fluids in inflammatory or tumorous circumstances and has been reported to be elevated in serum (FUJIMOTO; SAKAGUCHI; TAMAYA, 1999; GARZETTI; CIAVATTINI; LUCARINI, 1999; SANTIN; HERMONAT; PARHAM, 1999; QUEIROZ et al., 2012) and plasma (CLIFFORD et al., 2001; WERGIN et al., 2003) from patients with malignant tumors.

Recently it has been discussed in which blood compartment VEGF should be analyzed. *In vitro* experiments have indicated that plasma should be used since VEGF in serum is released from platelets in a time-dependent manner, making serum measurement unreliable (BANKS et al., 1998). It was investigated in this study the concentration of serum and plasma VEGF of healthy dogs in order to recommend optimal handling of biological samples for accurate measurement of VEGF.

Materials and methods

Animals

The present study was approved by the Bioethics Committee of the Faculty of Veterinary Medicine

and Animal Science of Universidade de São Paulo, receiving the protocol number 577/2004, and made with the free owner's consent who authorized in writing the participation of their animals in the study.

Blood samples were taken from thirty healthy intact male dogs presenting to the Veterinary Hospital of University of São Paulo/Brazil for elective orchiectomy. The mean age of dogs was four years (range from five months to eight years), the breed of dogs were four Labrador retriever, one Doberman and 25 German Shepherd.

Haematological parameters of all patients were recorded. The mean haemoglobin (hb) level was 17.1 ± 1.3 g/dL. The mean neutrophils count was $7.62 \times 10^3 + 1.87 \times 10^3/\mu\text{L}$. The mean platelets count was $2.04 \times 10^5 + 4.5 \times 10^4/\mu\text{L}$.

Measurement of VEGF

Blood samples of the dogs were collected into sterile EDTA tube for plasma analysis and into clot activator tubes for serum analysis. The tubes were centrifuged within 90 minutes of collection at 1400 xg for 10 minutes. The samples were stored at -80°C until assay. VEGF concentration was determined using the quantitative method (ELISA kit - Quantikine, R&D System) developed to measure the soluble VEGF levels (VEGF 121 and VEGF165) in various human fluids. The validation of the ELISA test to measure canine VEGF using human antibody was previously confirmed by Clifford et al. (2001) and Gentilini et al. (2005). The concentration values were calculated according to the manufacturer's protocol and obtained in picograms per milliliter (pg/mL).

Statistical analysis

Statistical analysis (SAS Institute, Cary, NC, USA) was performed using the ANOVA test and Pearson correlation.

Results

Serum VEGF level was 26.5 ± 13.3 pg/mL, significantly higher ($p = 0.0003$) compared with plasma VEGF, 11.7 ± 16.4 pg/mL.

Haematological parameters were within normal limits for the specie. There was a significant positive correlation between serum VEGF and platelets ($r = 0.37$, $p = 0.03$). There was a significant negative correlation between serum VEGF and hemoglobin ($r = -0.38$, $p = 0.03$) and between plasma VEGF and hemoglobin ($r = -0.34$, $p = 0.06$), but no significant.

Discussion

Vascular endothelial growth factor exists in four isoforms resulting from alternative exon splicing of its ribonucleic acid (RNA) transcript (HOUCK et al., 1991, TISCHER et al., 1991). Of these isoforms, only VEGF₁₂₁ and VEGF₁₆₅ are secreted in soluble form, with VEGF₁₆₅ being predominant soluble isoform (HOUCK et al., 1992).

The main isoforms of canine VEGF are VEGF₁₂₀, VEGF₁₆₄ and VEGF₁₈₈. The deduced amino acid sequence display only 4.8% changed residues compared to the human VEGF sequence (SCHEIDEGGER et al., 1999). We investigated the soluble isoform of VEGF in serum and plasma of health dogs using a quantitative method developed to measure the soluble VEGF levels in human fluids because of the structural similarity between the two species as mentioned by the above authors. Furthermore, the reliability of this assay for use in dogs was performed by Clifford et al. (2001) and Gentillini et al. (2005).

Serum provides the liquid portion of the blood without cells and clotting factors and, therefore, should contain proteins and other molecules that represent the whole body system. The cells and clotting factors must be removed from the blood sample by allowing adequate time for a clot to form. Most manufacturers of collections systems for serum samples recommend 30-60 min at room temperature for a clot to form and longer if the subject was taking any kind of anticoagulant at sample collection (ARZOUMANIAN, 2003).

Serum samples that are allowed to sit less than 30 min are likely to retain cellular elements and other contaminants impacting future analysis. Samples that

sit longer than 60 min are likely to experience lysis of cells in the clot, releasing cellular components not usually found in the serum samples (TIMMS et al., 2007). In this investigation the samples were allowed to sit 90 min, which may have caused cell lysis increasing serum VEGF when compared with plasma VEGF.

Kusumanto et al. (2003) conducted a study in thirteen healthy subjects and found that, in healthy people, 58% of VEGF in the bloodstream is the fraction of granulocytes. Maloney et al. (1998) founded that VEGF was secreted in vitro aggregation of platelet-rich plasma induced by thrombin, collagen, epinephrine and ADP. Furthermore, sérum VEGF levels were increased when compared with plasma. Salgado et al. (2001) observed a correlation between platelet count and sérum VEGF level in the healthy control. In the present investigation it was found a positive correlation between serum VEGF and the platelet in healthy dogs. These data corroborate with hypothesis of several researchers that platelets carriers VEGF to the circulating blood.

Plasma does not contain significant amounts of VEGF, indicating that VEGF measured from serum samples is released from the blood cells during the coagulation process. It should, therefore, be noted that variations in samples handling may affect the blood cell activation and, thus, the release of VEGF to serum (WARTIOVAARA et al., 1998). In this study the samples serum contained quantities of VEGF elevated when compared with plasma. Therefore, VEGF may have been released by cells lysis resulting from prolonged clotting and platelet activation.

One major stimulus for increased VEGF expression is hypoxia (HARRIS, 2002). Dunst et al. (2002) have reported an association between anaemia and elevated level of plasma VEGF in human patients. In the present paper, it was found that, as the quantity of hemoglobin was reduced, serum and plasma VEGF concentration was increased; suggesting that, even in the absence of anemia, the reduction in the amount of

hemoglobin can be a stimulus for release of VEGF in circulating blood.

VEGF has been implicated as an angiogenic factor in human and dogs with tumors through immunohistochemistry or blood concentration in serum and plasma samples, therefore, the outcomes are controversial. This study investigated VEGF concentration in plasma and serum of healthy dogs and revealed the plasma as the ideal fluid for measuring VEGF. Processing and handling of serum

can increase VEGF serum concentration. Further research should be undertaken comparing VEGF in these fluids of cancer patients.

Acknowledgments

This study was supported by grants from FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo - Processo N° 2004/08218-6) to which the authors are truly grateful.

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