Platelets and vascular endothelial growth factor (VEGF): A morphological and functional study

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Abstract

The growth of primary tumours beyond a critical mass is dependent on angiogenesis. The switch to the angiogenic phenotype involves changes in the local equilibrium of cytokines with either pro- or anti-angiogenic properties. Vascular Endothelial Growth Factor (VEGF) is one of the major positive regulators of tumour angiogenesis. Serum VEGF is, in cancer patients, correlated with worse prognosis. Recent evidence suggests that platelets are the main contributors of serum VEGF. We demonstrate, ultrastructurally and with immunofluorescence techniques, the alpha granule and membranous localisation of VEGF and provide further evidence for the role of platelets, both in healthy individuals as in patients with locally and advanced breast cancer, in the storage of circulating VEGF. We also demonstrate that, linear with tumoural progression, platelets accumulate more VEGF. Enhanced production in bone marrow platelet progenitors as well as endocytosis of circulating VEGF by platelets and/or megakaryocytes could explain the higher VEGF load in platelets from advanced cancer patients. This study provides further evidence for a role of platelets in transporting VEGF.

Abbreviations: EGF – epidermal growth factor; IL-6 – interleukin-6; PVEGF – plasma VEGF; PDGF – platelet derived growth factor; SVEGF – serum VEGF; SCCS – surface connected canalicular system; TGF-β – transforming growth factor-β; VEGF – vascular endothelial growth factor

Introduction

Vascular Endothelial Growth Factor (VEGF), also known as vascular endothelial permeability factor (VPF) and vasculotropin, is a disulphide linked homodimer of 34–42 kDa that shares structural homology with the PDGF (Platelet Derived Growth Factor)-family [1]. Four spliced variants have been described so far, containing 121, 165, 189 and 206 amino acid residues [2].

VEGF is an endothelial cell specific mitogen interacting with three tyrosine-kinase receptors, flt-1, flk-1 and flt-4 [3]. It regulates proliferation, differentiation and survival of endothelial cells [4], acting also as a chemoattractant for monocytes, mast cells and pericytes [5–7] and it enhances vascular permeability even more potently than histamine [10]. Several cytokines (EGF, TGF-β, IL-6) enhance VEGF mRNA expression [8, 9]. Recently, other endothelial growth factors related to VEGF have been identified in mammals, namely VEGF-B, VEGF-C, VEGF-D and Placental Growth Factor (PIGF) [11–13, 15].

VEGF is considered one of the major modulators of angiogenesis in a variety of physiological processes, like wound healing and embryonic development, and plays a role in the physiopathogenesis of diseases like diabetic retinopathy, psoriasis and tumour growth [8, 14, 15].

High serum levels of VEGF have been described in patients with cancer [16]. Although VEGF is produced and secreted by stromal and epithelial tumour cells [17, 18], circulating blood cells may also contribute to the overall VEGF level in the blood stream. Recent evidence suggests that platelets are the main contributors of serum VEGF [19–21]. Platelets and their precursor, the megakaryocyte, contain the mRNA and protein of VEGF [22], as well as the mRNA of both VEGF receptors, flt-1 and flk-1 [23].

It has been suggested that VEGF resides in the alpha granules of platelets [24]. However, no conclusive

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evidence regarding the ultrastructural localisation of VEGF has been documented. Remarkably, platelets of cancer patients contain higher VEGF levels, and hence cancer patients have higher blood levels than healthy controls [Benoy et al., submitted, 25]. How VEGF is regulated in megakaryocytes and platelets remains to be established.

This study aims at localising VEGF ultrastructurally, establishing evidence that platelets are major transporters of circulating VEGF and evaluating differences in platelet VEGF load between advanced cancer patients and healthy controls.

**Patients and methods**

**Enzyme-linked immunosorbent assay (ELISA)**

The study population consisted of 34 untreated metastatic breast cancer patients (20 patients had bone metastases, 17 patients had lung metastases and 11 patients had liver metastases. In 5, 6 and none of the patients, respectively, bone, lung or liver was the only involved site) and 21 patients with locoregional, untreated breast cancer. The control population consisted of 24 healthy individuals.

**Samples**

Venous blood samples were collected in serum separator tubes (type Vacutainer, Becton-Dickinson), and centrifuged at 2000 g for 5 min. Platelet counts were performed on EDTA-anticoagulated blood samples with an automated blood Coulter counter (GEN-S). Plasma samples were obtained after collecting venous blood in vacuum tubes, containing citrate, theophyllin, adenosine and dypiridamole (CTAD, Becton Dickinson Vacutainer Systems, Europe) in order to avoid platelet activation during sampling, and subsequent centrifugation at 2000 g for 5 min. Both serum and plasma samples were collected and stored at −80 °C.

**Laboratory assay**

Serum and plasma levels of VEGF165 were determined using an ELISA kit of R & D Systems (Minneapolis, Minnesota; Quantikine human VEGF). No cross-reactivity with other members of the VEGF-family is documented. Within assay reproducibility has been tested before [16]. Samples were assayed in duplicate.

**Immunofluorescence staining**

Platelets from one healthy individual and from three cancer patients (one patient with a metastatic breast carcinoma, one with metastatic cervix carcinoma and one with a metastatic caecum carcinoma) were studied.

**Platelet preparation**

Blood was collected in CTAD-tubes as mentioned previously. Platelet-rich plasma was obtained after centrifugation at 180 g for 10 min at room temperature (RT). The cells in the platelet rich plasma were counted as mentioned before to exclude contaminating leukocytes and red blood cells.

**Staining procedure**

Washed human platelets were, after fixation with 2% paraformaldehyde/0.2% glutaraldehyde, treated with 0.1% Triton for 3 min in order to render them permeable to the primary antibody. The platelets were washed with CTAD-buffer at 180 g for 10 min and suspended in 1/100 diluted primary goat anti-human VEGF antibody (R & D Systems, Minneapolis, Minnesota), for 30 min at 37 °C. This antibody recognizes specifically the 121 and 165 VEGF-isofoms. The cells were subsequently stained with 1/400 diluted secondary biotinylated rabbit anti-goat antibody (Dako, Glostrup, Denmark), for 30 min at RT. Finally, washing as described previously occurred followed by staining with 1/100 diluted streptavidin-texas red.

The primary antibody was omitted in control cells. The cells were subsequently viewed with a Zeiss Laser Scanning Epifluorescent Microscope 410 mounted on an Axiowert M 135 (Zeiss, Oberkochen, Germany). Examination of immune reactivity in platelets with a confocal laser scanning microscope yields, due to the small platelet size (measuring about 3 μm in diameter), images of higher quality compared with examination by conventional light microscope.

**Ultra-structural localisation of VEGF**

**Platelet preparation**

Platelets of a healthy individual were isolated as mentioned before.

**Embedding procedure**

Washed human platelets, after fixation with 2% paraformaldehyde/0.2% glutaraldehyde for 1 h, were subsequently used for imbedding in agar 0.3%. The cells were fixed with 2% paraformaldehyde/0.2% glutaraldehyde for 2 h. Serial dehydration and infiltrations with Unicyrl at −20 °C (British Biocell Int.) took place. After overnight infiltration with pure Unicyrl, polymerisation occurred at −20 °C using Philips LTD 15 W/05 lamps for 4 days, followed by a final curing period of 7 days at 4 °C [26].

**Post-embedding immunogold procedure**

Ultra-thin sections of the embedded tissue were made with a glass knife and sections were collected on formvar-coated nickel grids. The grids were preincubated for 30 min on 50-μl drops of Tris-buffered saline (TBS; 0.05 M, pH 7.6) containing 20% normal goat serum (Dako, Glostrup, Denmark). The grids were, after preincubation, incubated overnight in a humid chamber at +4 °C on 50-μl drops with 1/100 diluted primary rabbit anti-human VEGF antibody (Santa Cruz Biotechnology, Inc.). This antibody recognizes the 165, 189