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Association between Endothelial Progenitor Cells and Von Willebrand Factor in Astrocytic Glioma.

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ASSOCIATION BETWEEN ENDOTHELIAL PROGENITOR CELLS AND VON WILLEBRAND FACTOR IN ASTROCYTIC GLIOMA

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Abstract

Astrocytic gliomas are highly aggressive and lethal brain tumours that depend on angiogenesis for growth. The endothelial progenitor cell (EPCs) and von Willebrand factor (vWF) involved in the formation of new blood vessels in astrocytic glioma. Objectives: This study aimed to investigate the association between circulating and tissue resident EPCs with vWF in astrocytic glioma patients. Methods: Blood specimen and brain tissue biopsy were collected from a total of 22 astrocytic glioma patients admitted to Hospital Universiti Sains Malaysia. Circulating EPCs

(blood) and tissue resident EPCs (tissue biopsy) were characterized using EPC-specific markers, CD133 and VEGFR2 and quantified using fluorescence- activated cell sorting analysis and immunofluorescence microscopy, respectively. The plasma vWF was measured by using commercialized Elisa kit (Cusabio Biotech Co.,Ltd). Results: The mean percentage of circulating EPCs was (0.01 ± 0.01%), brain tumor tissue EPCs (0.48 ± 0.38%) and adjacent normal brain tissue EPCs (0.18 ± 0.23%). The mean plasma vWF was 9.23 ± 7.57%. Positive correlation was found between brain tumor EPCs and plasma vWF (Spearman's rho r = 0.45, p = 0.035). However no correlation was found between adjacent normal brain EPCs and plasma vWF. About 14 patients had (mild vWF level of > 5%), 8 patients had (moderate vWF level of 1-5%) and no patients had (severe vWF level of < 1%). The mean percentage of patients with mild vWF level was 12.48 ± 7.77% and moderate vWF level was 3.53 ± 1.32%. There was a significant correlation between circulating EPCs and patients with mild vWF level (Spearman's rho r = 0.63, p = 0.015). Conclusion: This study demonstrated that EPCs have significant positive association with vWF suggesting the homing of plasma vWF at the tumor site.

Keywords

Endothelial Progenitor Cell; Von Willebrand Factor; Astrocytic Glioma

1. Introduction

The von Willebrand Factor (vWF) is a high molecular weight plasma protein, produced and released by endothelial cells and circulates in the blood plasma [1-2]. The release of vWF from the endothelial cells happens when ABO blood group antigen particularly H antigen bind with N-linked glycan chains on vWF which occurs in post-Golgi compartment of endothelial cells. The release of vWF from endothelial cell storage sites can be stimulated by vasopressin analog (Gallinaro et. al; 2008). The vWF- glycoprotein is important for normal hemostasis that involved in inflammation and to regulate angiogenesis (Starke et. al; 2011). The vWF that principally found at the site of vascular injury regulates and initiates the formation of thrombus via platelet aggregation (Verma S. et. al; 2003). Study reported that thrombin stimulates tumor-platelet adhesion in vitro and metastasis in vivo (Nierodzik et. al; 1991). The vWF is a mediator that involved in adhesion of platelets to the collagen and hemostatic plug formation (Spiel et. al; 2008). The vWF not only function in the platelet adhesion to the vessel wall but also enhance the platelet to platelet interaction under high shear-stress conditions (Rumbaut et. al; 2010). These platelets interact with endothelial progenitor cells (EPCs) to repair the injured endothelium

(Frenette et. al; 1995). The vWF+ fractions expressed together with human blood and tumor EPCs (Choi et. al; 2012).

It has been shown that patients with microvascular dysfunction showed increased vWF/FVIII expression [9-10]. The astrocytic glioma patients with increased vWF expression showed severe damage in the microvasculature system (Hillen & Griifioen, 2007). The normal level of vWF/FVIII is 200 ng/ml (100%). The vWF/FVIII level was categorized into three levels of severity, severe (factor level less than 1%), moderate (factor level of 1-5%) and mild (factor level of >5%) (Borchielliizedni et. al; 1996). Previous studies has categorized the patient with factor level less than 1% as those who frequently bleed spontaneously and excessively after injuries, surgery. Patients with 1–5% of normal activity have moderately severe bleeding or have prolonged bleeding after injuries and patients with >5% of normal activity usually bleed only with surgery or trauma or prolonged bleeding after a serious injury, trauma or surgery (Borchiellini et. al; 1996).

The EPCs are being produced via bone marrow during the stage of postnatal life and enter into the blood stream circulations. EPCs in the blood stream act as a repair mechanism (Kheirandish-Gozal, et. al; 2010). The formation of tumour neo vessels occurs through luminal incorporation of EPCs. Significant reduction of EPCs showed impaired and delayed growth in tumour followed by reduction in tumour vessel density. During the vascular injury the circulating host derived endothelial cells are highly demanded for the neo angiogenesis. The tumour growth angio genic switch promotes the recruitment of EPCs from the bone marrow (Daniel J. et. al; 2007). Based on previous study, we believed that the expression of vWF determines the severity of damaged microvasculature system in astrocytic glioma patients and both vWF and EPCs involved in angiogenesis. Therefore in this study, the association between EPCs level (both circulating EPCs and resident EPCs) and vWF were investigated.

2. Methods

Patient Population

This study enrolled consecutively over a 3 year period (2012-2014). A total of 22 astrocytic glioma patients (WHO grade I to IV) from the Hospital Universiti Sains Malaysia (HUSM) were recruited in the study. Patients were identified by radiological appearance of astrocytic gliomas with subsequent histological confirmation. Only cases that were unruptured with no pre-surgical embolization were selected in the study. This study was approved by

Human Research Ethics Committee, Universiti Sains Malaysia (FWA Reg No: 00007718; IRB Reg. No: 00004494).

Blood Sampling

The peripheral venous blood was obtained from each patient during surgery for the analyses of circulating endothelial progenitor cells (cEPCs). At the time of surgery, 25ml peripheral venous blood was collected in EDTA tube via veni puncture from each patient. To minimize contamination with endothelial cells from the puncture wound of the vascular wall, the initial 5 ml blood samples drawn were discarded (Parney et. al; 2009). After collection, the blood samples were processed within an hour.

Flow Cytometry Analysis

The CEPCs in peripheral blood were quantitatively determined by using FACSCanto II flow cytometer (Becton Dickinson, USA). The peripheral mononuclear cells (PBMCs) were prepared by gradient centrifugation using Ficoll-Hypaque technique. PBMCs were pippetted into three 1.5 ml centrifuge tube with 106 cells each and added with 10 µl of FcR-blocking reagent for 10 minutes to inhibit any non-specific bindings. The cells were then incubated with 10 µl of phycoerythrin-conjugated (PE) anti-human CD133 monoclonal antibody (mAb) (Miltenyi Biotech, Bergisch Gladbach), 10 µl of allophycocyanin-conjugated (APC) anti-human VEGFR-2 mAb (Miltenyi Biotech, Bergisch Gladbach) and 10 µl of fluorescein Isothiocyanate (FITC) annexin (Miltenyi Biotech, Bergisch Gladbach) for 30 minutes at 4°C. As a measurement of negative controls the PE-, APC- and FITC- isotype-matched IgG1 (Miltenyi Biotech, Bergisch Gladbach) antibodies were used. The cells then washed three times with 1x Phosphate buffered saline (PBS) and re-suspended in 400 µl of FACS solution. The FLowJo_V10 software was used to analyse the data. About 50 000 events were collected and each probe were performed in triplicate. The percentage of CEPCs in peripheral blood was determined by 2D side-scatter/fluorescence dot-plot analysis. The CEPCs were expressed as percentage of total PBMCs in each patient (Rafat N. et. al; 2010).

Tissue Sampling

Brain tumour and normal adjacent tissue microsurgical specimens were obtained from each patient (n=22) after the surgery for the analyses of tissue resident EPCs.

Immunofluorescence Microscopy

The total percentage of resident EPCs in the brain tumour and normal adjacent brain specimen was characterized using immunohistochemistry staining method. Tissue biopsies were fixed in paraformaldehyde 10% for at least 48 hours and were cut into 3 sections with an interval and thickness of 4 mm. The tissue sections were processed in automated tissue processor (Leica TP 1020, Germany) and embedded in wax paraffin block. Tissue blocks were cut with a microtome (Microm HM 325 Rotary Microtome, Germany) in a thickness of 3 µm. First, the tissue sections were deparaffinized by using two changes of xylene, followed by xylene: ethanol in ratio 1:1 and rehydration with two changes of absolute, 95% and 70% ethanol for 3 minutes each. Tissue sections were then rinsed under running cold tab water and added with enough drops of 0.1% Triton x-100/PBS for 10 minutes. Next the tissues were washed with 1x PBS for 3 times and added with 0.5% BSA in PBS for 5 minutes.

The tissue sections were stained with PE anti-human CD133 (Miltenyi Biotech, Bergisch Gladbach) and FITC anti-human VEGFR2 (Bioss, Woburn Massachusetts). After overnight incubation at 4°C in a dark humid chamber, the tissue sections were washed with 1x PBS for 3 times and counterstaining with 4',6-diamidino-2-phenylindole (DAPI) were performed for 30 minutes. The slides were then washed with 1x PBS for 3 times and mounted in mounting medium and then analysed using BX41 Olympus microscope at 200x magnification. The images were captured and merged to see the expression of the markers. Images of a total of 24 fields were captured for brain tumour sample and another 24 fields for adjacent normal brain per patient. Each field consisted 0.57 mm^2. Therefore, the total tissue areas which had been investigated to determine the resident EPCs for both tumour and adjacent normal brain in 22 patients were 606.50 mm^2. The percentages of total tissue area stained with resident EPCs were analysed using Image J software 1.45s.

Plasma vWF/FVIII

Plasma concentration of vWF was quantitatively determined by using vWF/FVIII Elisa kit (Cusabio Biotech Co., Ltd). The standard was prepared with known concentration as described in manufacture protocol. The standard vial was centrifuged at 6000 rpm for 30 s. Then the standard was reconstituted with 1.0 ml of sample diluents. This reconstitution produces a stock solution of 400 ng/ml for vWF/FVIII marker. The serial dilutions were performed by 250

ul of sample diluents added to 1.5 ml micro centrifuge tubes and mixed with 250 ul of standard stock solution of vWF/FVIII respectively. The series of dilution were prepared for the concentration of (400 ng/ml, 200 ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml and 6.25 ng/ml). The blank sample added with sample diluents for 0 ng/ml concentration. 250 µl of Sample diluent into each tube (S0-S6) were pipetted. After the standards dilution steps, 100 ul of these standards and samples added into each of a pair of adjacent wells. The covered wells were then incubated for 2 hours at 370C. Then, the liquid from each well was removed without washing. Then 100 ul of Biotin-antibody working solution for each well was added and incubated for 1 hour at 370C. After incubation the wells were washed for three times. The HRP-avidin working solution (100 ul) was added to each well, then the microtiter plate was covered with new adhesive strip and incubated for 1 hour at 370C. The wells were washed again for five times. 90 ul of TMB substrate was added to each well and incubated for 10-30 minutes at 370C. Finally 50 ul of stop solution was added to each well and the optical density of each well was determined by using a microplate reader set to 450 nm within 30 minutes.

The duplicate reading for each standard, control and sample were averaged and subtracted with the average zero standard optical density. The standard curve was plotted and data was analysed.

Statistical Analysis

All data were presented as mean ± SD. The Spearman's rho correlation test was performed. The statistical significance was determined at $p < 0.05$. SPSS software version 20.0 (IBM, Armonk, NY, USA) was used for all the analyses.

3. Results

Participant Characteristics

Twenty two patients with astrocytic glioma were enrolled in this study including (WHO grade IV n= 9, grade III n = 8, grade II n = 2, grade I n = 3). The male patients (n = 16) and female patients (n = 6) were included in the study. The mean age of the patients was 44.18 ± 14.79 years. The clinical characteristics of patients are shown in Table 1.

Level of Plasma vWF/FVIII

The vWF/FVIII levels among all the glioma patients were lower than the normal range (100%). About 14 patients with (mild factor level of >5%), 8 patients with (moderate factor level of 1-5%) and no patients had (severe factor level of < 1%) was found in the study. The mean percentage of patient with mild vWF/FVIII level was 12.48 ± 7.77 % and moderate vWF/FVIII factor level was 3.53 ± 1.32%.

Association between Plasma vWF/FVIII and Brain Tumor EPCs

The mean plasma vWF/FVIII was 18.45±15.15 ng/ml. The mean percentage of brain tumor tissue EPCs was 0.48 ± 0.38% and adjacent normal brain tissue EPCs was (0.18 ± 0.23%). Spearman's rho correlation test was performed between plasma vWF/FVIII and brain tumor EPCs to determine the association. The result showed that there was a significant correlation between plasma vWF/FVIII and brain tumor EPCs (Spearman's rho r = 0.452, p = 0.035), Fig 1. However no correlation was found between plasma vWF/FVIII with adjacent normal brain (Spearman's rho r = 0. 210, p = 0. 349).

Association between Plasma vWF/FVIII Level and cEPCs

The mean percentage of CEPCs was (0.01 ± 0.01%) Those patients with mild vWF/FVIII level was significantly correlated with CEPCs (Spearman's rho r = 0.63, p = 0.015), Fig 2.

Table 1: *Socio-Demographic and Clinical Characteristic of Respondents*

Characteristics	n (%)
Age (mean)	44.2
Gender	
Male	16 (70.0)
Female	6 (30.0)
Astrocytic glioma diagnosis	
Glioblastoma multiformae	9 (40.9)
WHO grade IV	
Anaplastic WHO grade III	8 (36.4)

Diffuse WHO grade II	2 (09.1)
Pilocytic WHO grade I	3 (13.6)

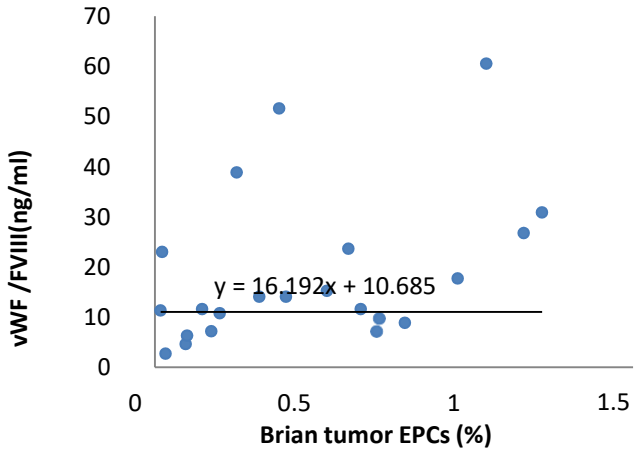

Figure 1: *Correlation between Vwf/Fviii and Brain Tumor Epcs of Astrocytic Glioma Patients*

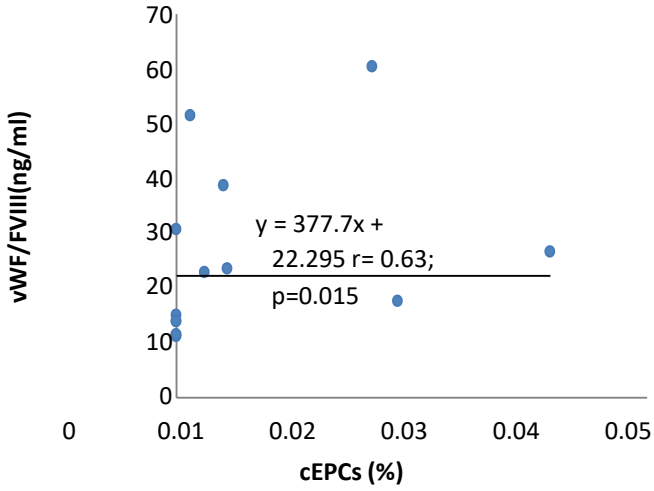

Figure 2: *Correlation between vWF/FVIII and cEPCs of glioma patients with mild vWF/FVIII level*

4. Discussion

Endothelial vWF involved in the vessel formation and vascular development (Gallinaro et. al; 2008). In current study, the data showed that the plasma vWF/FVIII associated with the brain tumor EPCs and not with the adjacent normal brain EPCs in the astrocytic glioma. This

study also found vWF/FVIII levels among all the glioma patients were lower than the normal range. Therefore we postulate that the glioma tumours have impairment in the vasculature system rather than in the normal brain tissue vascular system. This might be due to the aggressive mass of tumour progression that may induce abnormal vasoconstriction and vasodilatation of the tumour vascular systems that injure the blood vessels at the tumour site. This was supported by previous studies which found that newly formed blood vessels in the tumour appear to be less disorganised, tortuous, dilated, leaky and hemorrhagic [16-19].

It was also found that 14 patients with vWF/FVIII (mild factor level of >5%), 8 patients with (moderate factor level of 1-5%) and no patients had (severe factor level of < 1%) in this study. Jurasz et al, found that the vWF significantly increased in the plasma of cancer patients and suggested that the possibilities included the patients might have damaged endothelium, activation and aggregation of platelet or angiogenesis of the tumour (Jurasz et. al; 2001). In this study, the patient with mild level of vWF/FVIII had a significant correlation between plasma vWF/FVIII and cEPCs. This result suggested that the vWF/FVIII being released more frequently from the tumour endothelium and circulated in the circulating system to attract more CEPC to be homing towards the tumour site to repair the injured endothelium. This finding was supported by previous studies which reported the vWF being produced and released by endothelial cells and circulates in the blood plasma [1-2]. The study also found that vWF played roles in the platelets adhesion to the vessel wall and enhanced the platelet to platelet interaction (Rumbaut et. al; 2010). These platelets then interacted with EPCs that mediated by P-selectin on EPCs and P-selectin glycoprotein ligand-1 (PSGL-1) on platelets (Frenette et. al; 1995 ;Paul S. et. al; 2000). This interaction enhanced the homing of the CEPCs towards the tumor site. This phenomenon might also indirectly enhanced the angiogenesis of the tumour as endothelial vWF involved in the blood vessel formation (Gallinaro et. al; 2008). Moreover both extracellular and intracellular vWF/FVIII regulates the angiogenesis processes (Starke et. al; 2011).

In another study which investigated the relationship between vWF and tumor cell induced platelet aggregation found that the vWF involved in the tumour platelet-aggregatory. Multimericprotein vWF mainly present in the plasma, platelets, endothelial cells, and in the sub endothelium and this large multimericprotein function in major adhesion that regulates platelet binding to the vascular wall. This occurs through the interaction of vWF with platelet GPIb and GPIIb/IIIa receptors. The researchers suggest that tumour cells enhance the aggregation of the platelets by upregulating platelet surface GPIIb/IIIa receptors to the site of vascular injury that

have pre-existing platelet adhering on vascular damaged site (Jurasz et. al; 2001). The inhibition of endothelial vWF expression retards the signalling of VEGF-2 for the endothelial cells proliferation, migration and formation of new vessels by decreasing integrin v 3 levels and increasing angiopoietin (Ang)–2 releases (Starke et. al; 2011). In another study on human brain tumours and peripheral blood showed CD133+ EPCs co express together with vWF. The study demonstrated that the human brain tumours EPCs have angio genic properties. The endothelial cells also involved in vWF expression compared with CD133- tumor EPCs. CD133+ tumour EPCs express vWF fractions as (80.6 ± 5.8%) and in CD133+ blood EPC (93.0 ± 2.4%), but only (5.5 ± 3.3%) in CD133- tumor cells. The result were significant with $P < 0.001$ (Choi et. al; 2012). Moreover the vWF also has capabilities to enhance the embryonic EPCs to differentiate into matured endothelial cells. Therefore the vWF plays a vital role in repairing the injured tumour endothelium and enhance the angiogenesis at the tumour site (Vajkoczy et. al; 2003) .

5. Conclusion

We investigated on association between EPCs and vWF in astrocytic glioma. The tumor EPCs were positively correlated with plasma vWF/FVIII and no correlation was observed with normal adjacent brain tumor EPCs. The patient with mild levels of vWF/FVIII had a significant positive correlation between plasma vWF/FVIII and cEPCs.

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