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Research Article

Association Between Macrophages with Angiogenic Cells and Microvascular Dysfunction in Astrocytic Glioma

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Abstract

Objectives: Astrocytic gliomas are the most common primary brain tumours that developed from glial origin. Macrophages are the predominant inflammatory cells in infiltrating gliomas.

The study aimed to investigate the association between circulating macrophages with tissue resident angiogenic cells and microvascular dysfunction in astrocytic glioma.

Methods: A total of 22 astrocytic glioma patients were consented from Hospital Universiti Sains Malaysia. Tumour (n=22) were sliced and stained with CD133+ and VEGFA+ angiogenic markers and counter stained with DAPI. The Spearman rho's test was used for the data analysis. The plasma CD68, macrophage level and von Willebrand factor level or factor VIII level (vWF/ FVIII) was measured using Elisa Kit (CUSABIO BIOTECH CO., LTD).

Results: The mean plasma CD68 macrophages was 12.48±19.98 pg/ml. The mean percentage of brain tumour tissue angiogenic cells was (1.26±0.95%), adjacent normal brain tissue angiogenic cells was (0.72±0.68%). Spearman's rho correlation test showed a significant correlation between brain tumour angiogenic cells and plasma CD68 macrophages (n=22) (Spearman's rho, r=0.43, p=0.047). However no correlation was observed between plasma CD68 macrophages with adjacent normal brain (Spearman's rho r =0. 019, p=0. 932). In this study vWF/FVIII level was analysed and 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. The mean vWF/FVIII percentage patient with mild level was 12.48±7.77 % and moderate vWF/FVIII factor level was 3.53±1.32%. The spearman's rho correlation test showed a significant correlation between patient with moderate vWF/FVIII level with plasma CD68 macrophages (Spearman's rho r=0.73, p=0.041).

Conclusion: Circulating macrophages associated with brain tumour angiogenic cells and vWF/FVIII of astrocytic glioma. Thus targeting these parameters in the treatment of glioma might be useful.

Keywords: Angiogenic cells, astrocytic glioma, brain tumour, macrophages, microvascular dysfunction

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Gliomas that found at intracranial site accounts for 30 to 45% of the tumour in the human. The bone marrow cells contribute towards the microglial brain population such as macrophages.[1] Besides endothelial progenitor cells (EPCs), the local immune microenvironment within glioma is dependent on both glioma cells and infiltrating inflammatory cells. Some data suggest that microglia (the brain's resident antigen-presenting cells) and/or macrophages and neutrophils are the predominant inflammatory cells in infiltrating gliomas.[2-6]

In a study among the lung cancer patients and normal patients the macrophage (CD163+) level was higher compared to non-malignancy patients and about half of these macrophages (CD163+) were positive for CD68+ markers indicating its infiltrating properties. The study also suggests that the CD163+ macrophages undergo transition from M1 to M2 phenotype that causes no functional macrophage against the tumour and eventually makes the tumour into a malignant state.[7] The vascular endothelial growth factor as one of the tumour-derived chemoattractants responsible in the recruitment of tumour associated microglia/macrophages. The study reported that the tumour associated microglia/macrophages that normally found surrounding the glioma initiating cells associated positively with the grade of the tumour and glioma initiating cell numbers. The recruitment and infiltration level of macrophages into glioma initiating cells was higher compared to adhesive glioma cells. The CD68 marker which is responsible to stain the tumour associated microglia/macrophages found to be increased along with tumour malignancy following order of 7.9% in WHO grade II ,19.7% in WHO grade III and 29.2% in WHO grade IV.[8]

In this study the angiogenic cells in the tissue resident was determined using two markers (CD133+ and VEGFA+) expression. In the previous staining of CD133+ cells in gliomas vary from complete lack of immunoreactivity to single cells expression or cell clusters staining. The CD133+ cells mean proportion of staining with WHO grade II (0.9%), III (7.2%) and IV (16.7%) were determined. The CD133 correlated with CD68 with r value of 0.730; p=0.01 and also correlated with glioma initiating cells with Iba1 marker expression, r=0.634; p=0.01. Both glioma initiating cells and tumour associated microglia/macrophages located at nest or cords in tumour cores and margins and also around microvessels and mostly found in perivascular region. The tumour associated microglia/macrophages regulated by VEGF-A and neurotensin. The tumour associated microglia/macrophages mainly found around glioma initiating cells which responsible to secrete the cytokines such as VEGF-A. The VEGF-A help to promote more recruitment of tumour associated microglia/macrophages to enhance the angiogenesis and to promote their immunosuppressive capacity.[8]

The vWF is glycoprotein that involved in inflammation and angiogenesis[9] that found at the site of vascular injury site. [10] Endothelial vWF function in vessel formation and vascular development.[11] Since the newly formed blood vessels at the tumour site appear to be less disorganised, tortuous, dilated, leaky and hemorrhagic,[12-15] we postulate vWF play important role in repairing the injured endothelium and enhance the recruitment of macrophages, CD68.

The current study aimed to investigate the association between macrophages with angiogenic cells and microvascular dysfunction in astrocytic glioma. To our knowledge none has investigated the marcrophage inflammatory cells with angiogenic cells and microvascular dysfunction in astrocytic glioma. Therefore, in this present study, we are investigating the involvement of inflammatory cells in gliomas in relation to angiogenic cells and microvascular dysfunction in astrocytic glioma as it influences the pathophysiology of tumour cells.

Methods

Patient Population

Astrocytic glioma patients (WHO grade I to IV) from the Hospital of Universiti Sains Malaysia (HUSM) that were recruited in the study. The sampling was done over a 3-year period (2012–2014). Twenty two patients with confirmed diagnosis of astrocytic gliomas with histological confirmation were enrolled in the study.

Tissue sampling

Microsurgical specimens of brain tumour and adjacent normal brain tissue from the same 22 astrocytic glioma patients were obtained from each patient after the surgery for the analyses of tissue EPCs cells with CD133+ and VEGFRA+ markers. Tissue biopsy were fixed in 10% paraformaldehyde and analysed for immunofluorescence microscopy.

Immunofluorescence Microscopy

The percentage of CD133+VEGFA+ in the tumour specimens was characterized using immunofluorescence microscopy method. Tissue biopsy was fixed in paraformaldehyde 10% and then brain tissue samples and adjacent normal brain tissue were cut thickness of 4 mm. The tissue then processed in tissue processor (Leica TP 1020, Germany) and embedded in paraffin block. Tissue blocks were cut serial dissections with a microtome (Microm HM 325 Rotary Microtome, Germany) for thickness of 3 µm. The tissue then were deparaffinized with two changes of xylene, then by xylene 1:1 with ethanol and rehydration with two changes of absolute, 95% and 70% ethanol each in 3 minutes. Then

the tissue with running cold tab water was rinsed. The tissue was applied with drops of 0.1% Triton x-100/PBS for 10 minutes and washed 3 times with 1x PBS. Finally the tissue added with PBS/BSA 0.5% for 5 minutes.

The tissue sections then stained with two antibodies; Phycoerythrin-conjugated (PE) anti-human CD133 monoclonal antibody (mAb) and Fluorescein Isothiocyanate (FITC) anti-human VEGFA+ (Bioss, Woburn Massachusetts) with overnight in a dark humid incubation chamber at temperature of 4°C. The tissue then were washed with 1X PBS for 3 times and counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 30 minutes. The slides washed with 1X PBS for 3 times and mounted in prolong antifade (life technologies) mounting medium and assessed through BX41 Olympus microscopy. The images were captured at 200x magnifications and merged to find the co expression of the two markers. Each sample of brain tumour about 606.50 mm^2 of the tissue area including brain tumour and adjacent normal tissue were investigated. The percentages of EPCs in the tumour and adjacent normal brain (CD133+VEGFA+) were determined using image J software 1.45s.

ELISA

Plasma was prepared from the blood collected in EDTA tube. The blood was centrifuged for 15 minutes at 1000g within 30 minutes of collection. The sample then was stored at -800C. The sample was centrifuged again after thawing before the assay.

The amounts of circulating inflammatory cells from plasma were measured using ELISA kits. The ELISA kits for CD68 (marker for macrophages) and ELISA kits for vWF (marker for microvascular dysfunction) were used according to manufacturer's instructions. Each sample was performed in duplicate.

Plasma concentration of CD68 was quantitatively determined by using CD68 Elisa kit (CUSABIO BIOTECH CO.,LTD). The standard prepared with known concentration. The standard vial was centrifuged at 6000 rpm for 30 s. The standard reconstituted with 1.0 ml of sample diluents. This reconstitution produces a stock solution of 1000pg/ml for CD68 marker. Meanwhile the reconstitution for vWF/FVIII produces a stock solution of 400 ng/ml marker. The standard was allowed to sit for a minimum of 15 minutes with gentle and uniform agitation by pipette with 1 ml measuring range prior to making serial dilutions. The serial dilutions were performed by 250 ul of sample diluents added to 1.5ml microcentrifuge tubes and mixed with 250 ul of standard stock solution. The series of dilution were prepared for the concentration of (1000pg/ml, 500pg/ml, 250 pg/ml, 125 pg/ml, 31.25 pg/ml, and 15.63 pg/ml) for CD68 marker. The series of dilution were prepared for the concentration of (400ng/ml , 200ng/ml, 100 ng/ml, 50 ng/ml, 25 ng/ml, 12.5 ng/ml and 6.25 ng/ml) for vWF marker. The blank sample added with sample diluents for 0 ng/ml concentration.

250 µl of Sample Diluent into each tube (S0-S6) were pipetted. The stock solution to produce a 2-fold dilution series was prepared. Each tube was mixed thoroughly before the next transfer. The undiluted standard serves as the high standard with 1000 pg/ml for CD68 concentration and the sample diluents serves as zero standard with 0 pg/ml concentration. The standard preparation was used within 4 hours and will be discarded after use.

After the standards dilutions were done, 100ul of these standards and samples added into each pair of adjacent wells. The covered wells were incubated for 2 hours at 370C. Then, the liquid from each well were removed without washing. Then 100 ul of Biotin-antibody working solution of each well were added and incubated for 1 hour at 370C. After incubation the wells are washed for three times. The 200 ul of wash buffer was added and let it stand for 2 minutes, and then liquid was removed by flicking the plate on a paper towel. The HRP-avidin working solution (100ul) added to each well, the microtiter plate covered with new adhesive strip and incubated for 1 hour at 370C. The wells were washed again for five times. 90ul of TMB substrate were added to each well and incubated for 10-30 minutes at 37 °C. The plate kept away from drafts and other temperature fluctuations in the dark. Finally 50 ul of stop solution were added to each well and the optical density of each well were 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. The normal level of vWF/FVIII is 200 ng/ml (100%). The vWF/FVIII level was categorised into three levels of severity, severe (factor level less than 1%), moderate (factor level of 1-5%) and mild (factor level of >5%).[16]

Statistical Analysis

All data are presented as means±SDs. The correlation analyses Spearman rho's correlation test was used. Statistical significance was set at $p<0.05$. All analyses were performed using SPSS software version 20.0.

Results

Participant Characteristics

Twenty two patients with astrocytic glioma were recruited in this study which included all grades of glioma

(WHO grade IV, n=9, grade III, n=8, grade II, n=2, grade I, n=3). Types of diagnosis were pilocytic astrocytoma, diffuse fibrillary astrocytoma, diffuse astrocytoma, anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic ependymoma, anaplastic gemistocytic astrocytoma, glioblastoma with oligodendroglioma component, gliosarcoma and glioblastoma multiformae. Socio-demographic and clinical characteristics of patient's are shown in Table 1. All data are presented as median ±SDs.

The present study showed that the mean percentage of brain tumour tissue angiogenic cells was (1.25±0.21%), adjacent normal brain tissue angiogenic cells was (0.74±0.15%). The mean plasma CD68 was 12.48±4.26 pg/ml. Spearman's rho correlation test was performed between plasma CD68 with tissue resident angiogenic cells. There was a significant association between brain tumour angiogenic cells and plasma macrophage, CD68 (Spearman's rho, r=0.43, p=0.047), Figure 1. However no correlation was found between plasma CD68 with adjacent normal brain (Spearman's rho r =0. 019, p=0. 932).

Table 1. Socio-demographic and clinical characteristics of respondents

Characteristics	n (%)
Age	
≤40 years	8 (40.0)
>40 years	14 (60.0)
Gender	
Male	16 (70.0)
Female	6 (30.0)
Astrocytic glioma diagnosis	
Glioblastoma multiformae WHO grade IV	9 (40.9)
Anaplastic WHO grade III	8 (36.4)
Diffuse WHO grade II	2 (09.1)
Pilocytic WHO grade I	3 (13.6)

In this study vWF/FVIII level was analysed and about 14 patients had (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. The mean vWF/FVIII percentage patient with mild level was 12.48±7.77 % and moderate vWF/FVIII factor level was 3.53±1.32%. The spearman's rho correlation test showed a significant correlation between patient with moderate vWF/FVIII level with plasma CD68 macrophages (Spearman's rho r = 0.73, p=0.041), Figure 2.

Discussion

The macrophage marker CD68 is used to detect the tumour-infiltrating macrophages.[17] The presence study found a significant correlation between plasma macrophage CD68 and brain tumour angiogenic cells. However no correlation was found between plasma macrophage CD68 with the normal brain tissue angiogenic cells. This supported by previous study that found the presence of macrophage clusters surrounding infiltrating glioma tumour cells that suggest the recruitment of macrophages towards the tumour site.[18] The macrophages correlated with tumour angiogenic cells could be explained by previous research studies that reported the thymidine phosphorylase positive cells that originated from macrophage CD68, involved in angiogenesis processes by stimulating the tumour cells to secrete vascular endothelial growth factor.[19] In another study it was found the population of macrophages abundantly present in glioma tumour compared to normal brain parenchyma.[20] Predominant population of microglia/macrophages identified in the glioma tumour tissue compared to normal brain tissue suggesting the macrophages have capabilities to infiltrate into the glioma tissue.[21] Other possibilities could be the ABCB1A, ABCG2 and ABCC4 transporters that found in stromal and brain side population for the efflux activities that found to be lacking in the macrophages that cause the accumula-

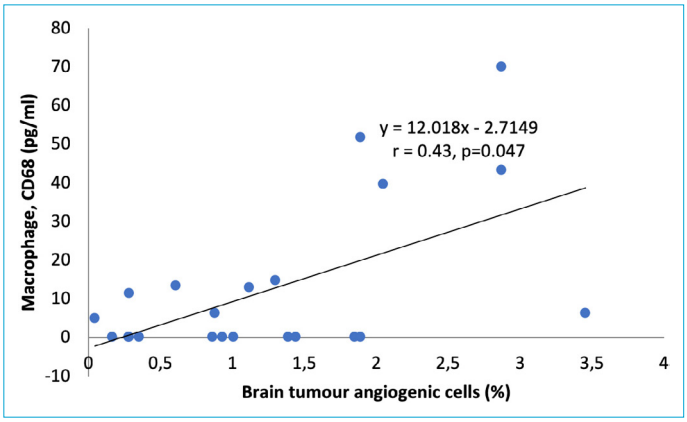

Figure 1. Correlation between macrophage (CD68) and brain tumour angiogenic cells of astrocytic glioma patients.

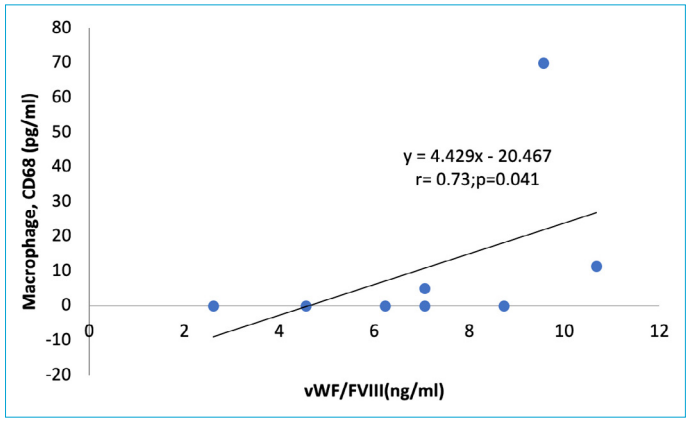

Figure 2. Correlation between macrophage (CD68) and vWF/FVIII of astrocytic glioma patients with moderate vWF/FVIII level.

tion of macrophages towards the glioma tumour site compared to parenchyma of the normal brain.[20]

This study also found among the moderate vWF/FVIII level patients, there was a significant association between macrophage (CD68) and vWF/FVIII level. The previous study has reported the patients with moderate level of vWF/FVIII tends to have have moderately severe bleeding or have prolonged bleeding.[16] We postulate that the glioma patients having microvascular damage or dysfunction at the tumour site and this phenomenon makes the recruitment of macrophages, CD68 towards damaged endothelium at the tumour site to repair the injured blood vessels by producing the inflammation. We also postulate that the macrophages at the damaged endothelium might infiltrate into the tumour tissues through the damaged endothelium or spilt vessels to eradicate the tumour cells. This finding supported by previous study that found the local immune microenvironment within glioma is dependent on both glioma cells and infiltrating inflammatory cells especially the macrophages. Some data suggest that microglia (the brain's resident antigen-presenting cells) and/or macrophages is the predominant inflammatory cells infiltrating the gliomas.[2-6] The macrophages of human gliomas act as a predominant immune subset that produce innate immune responses that activate antitumour effectors T cells against malignant human gliomas.[21] The previous study found the presence of macrophages in glioma tumours account for 35% of the glioma tumour mass.[17-18] The role of the macrophages might involved in tumour eradication.[18] Macrophages involves in suppression of the tumour growth by inducing antitumour immune response. However the immune responses of macrophages often inactivated by glioma cells through secretion of immunosuppressive factors that leads to tumour progressions.[22]

Conclusion

This study found that the circulating macrophages correlated with brain tumour angiogenic cells and plasma vWF/FVIII in astrocytic glioma. Therefore the treatment that consider these parameters might help in providing better treatment for the patients.

Disclosures

Ethics Committee Approval: Ethical approval was obtained from the Human Research Ethics Committee, Universiti Sains Malaysia (FWA Reg No: 00007718; IRB Reg. No: 00004494).

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – P.D.; Design – P.D.; Supervision – N.N.N., N.W.A., K.N., Y.C.K., K.R.; Data collection &/or processing – P.D.; Analysis and/or interpretation – P.D., N.N.N., N.W.A., K.N., Y.C.K.; Literature search – P.D.; Writing – P.D.; Critical review – P.D., N.N.N.

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