

Profile of VEGF Secretion in Human Peripheral Blood Mononuclear Cells *In vitro*

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Abstract: Peripheral Blood Mononuclear Cells (PBMCs) are key regulators of inflammation. Vascular Endothelial Growth Factor (VEGF) is a cytokine which is produced by a number of cells and has an important role in inflammation. In this study, the patterns of VEGF expression by human PBMCs were assessed *in vitro* using Phytohemagglutinin (PHA), Phorbol Myristate Acetate (PMA) and Lypopolysaccharide (LPS) as VEGF inducers. Human PBMCs were cultured in complete RPMI-1640 medium. Then, the cells were seeded at a density of 10^6 cells mL^{-1} and subsequently were incubated with PMA ($0-25 \text{ ng mL}^{-1}$), PHA ($0-10 \mu\text{g mL}^{-1}$) or LPS ($0-4 \mu\text{g mL}^{-1}$) for 48 h. The level of VEGF secreted in the cell culture supernatants was measured with enzyme-linked immunosorbent assay kits (R and D systems). Human PBMCs cultured without any stimulus, produced detectable amount of VEGF after 48 h incubation. PMA significantly reduced while PHA/LPS significantly increased VEGF production in human PBMCs dose-dependently. The results show that PMA, PHA and LPS have different effects on VEGF production by PBMCs.

Key words: VEGF, profile, human, PBMCs, therapeutic

INTRODUCTION

Inflammation plays an important role in various diseases, such as multiple sclerosis, rheumatoid arthritis and some cardiovascular disorders (Abrahamsson *et al.*, 2013; Van Herwijnen *et al.*, 2013; Yardim-Akaydin *et al.*, 2013). Peripheral Blood Mononuclear Cells (PBMCs) are key regulators of inflammation (Downer *et al.*, 2013; Santer *et al.*, 2012). The inflammatory effects of PBMCs are accomplished through some factors especially inflammatory cytokines (Youbare *et al.*, 2013; Gola *et al.*, 2013; Azimi-Nezhad *et al.*, 2013). Vascular Endothelial Growth Factor (VEGF) is a cytokine which is produced by a number of cells and has a key role in inflammation (Shaik-Dasthagirisahab *et al.*, 2013; Yuan *et al.*, 2013). Also, the increased serum level of VEGF in inflammatory diseases has been reported (Karatolios *et al.*, 2012; Park *et al.*, 2012). The expression of VEGF-A mRNA from PBMCs of thromboangitis obliterans has been shown (Fazeli *et al.*, 2013).

Moreover, expression the of VEGF-145 isoform mRNA and its relations to cellular adhesion molecules has been discovered (Azimi-Nezhad *et al.*, 2013).

Furthermore, an association between the increase in risk of Multiple Sclerosis (MS) exacerbation and enhanced disease activity with VEGF production by CD4⁺T cells has been revealed (Correale *et al.*, 2012). In addition, high VEGF Levels in PBMCs and pulmonary edema fluid of acute hantavirus pulmonary syndrome

patients has been detected (Gavrilovskaya *et al.*, 2012). Besides conditioned medium of oral carcinoma tumor cells induced production of VEGF by normal human PBMCs (Franca *et al.*, 2011).

Moreover, the elevated expression of VEGF in PHA-activated T cells by human dental pulp-derived stem cells has been shown (Demircan *et al.*, 2011).

Also, intrauterine administration of PBMCs in pregnant mouse increased the endometrial VEGF expression and pregnancy rate (Yu *et al.*, 2013).

Effect of some anti-inflammatory agents has in part attributed to their suppressive effect on VEGF secretion (Palmieri *et al.*, 2011; Gallelli *et al.*, 2013). Furthermore, the preventive effect of a number VEGF-inhibitors in autoimmunity has been shown (Seabrook *et al.*, 2010).

Due to important role of PBMCs and also VEGF in a number of physiologic and pathologic conditions, such as inflammatory and autoimmunity states (Sendergaard *et al.*, 2013; Sohn *et al.*, 2013), determination the expression profile of VEGF in PBMCs could be valuable in screening of VEGF enhancers/inhibitors, selection the ideal drugs with modulatory effects on VEGF and designing of novel drugs to target VEGF response in related disorders. In this study, the patterns of VEGF expression by human PBMCs were assessed *in vitro* using Phytohemagglutinin (PHA), Phorbol Myristate Acetate (PMA) and Lypopolysaccharide (LPS) as VEGF inducers.

MATERIALS AND METHODS

Reagents: RPMI-1640 medium, penicillin, streptomycin, Ficoll-hypaque, Lypopolysaccharide (LPS), Phorbol Myristate Acetate (PMA) and Trypan Blue (TB) were from sigma (USA). Fetal Calf Serum (FCS) was from Gibco (USA). Microtiter plates, flasks and tubes were from Nunc (Falcon, USA). VEGF standard ELISA kit was obtained from R and D company (USA).

PBMCs isolation: PBMCs from the venous blood of healthy adults volunteers were isolated by ficoll-hypaque-gradient centrifugation. Subsequently, the cells were washed three times in Phosphate Buffer Saline (PBS). Then the cells resuspended in RPMI-1640 medium supplemented with 10% FCS and were incubated in 5% CO₂ at 37°C.

Cell culture and treatment: The method has been described in detail elsewhere (Hajighasemi and Mirshafiey, 2010). Briefly, the human PBMCs were cultured in RPMI-1640 medium supplemented with 10% FCS, penicillin (100 IU mL⁻¹) and streptomycin (100 µg mL⁻¹) at 37°C in 5% CO₂. The cells were seeded at a density of 10⁶ cell mL⁻¹ and then incubated with different concentrations of PMA (0-25 ng mL⁻¹), PHA (0-10 µg mL⁻¹) or LPS (0-4 µg mL⁻¹) for 48 h. The supernatants of cell cultures were collected, centrifuged and stored at -80°C for next experiments. All experiments were done in triplicate.

Evaluation of VEGF production by ELISA: The amount of VEGF secreted in the cell culture supernatants by human PBMCs was measured with the Quantikine human VEGF ELISA kits (R and D systems) according to the manufacturer's instructions. This assay uses the quantitative sandwich enzyme immunoassay technique. Complete RPMI medium was used as control and human recombinant VEGF165 was employed as standard for drawing the standard curves.

Statistical analysis: VEGF level quantification in cell-conditioned media was performed in three independent experiments and the results were expressed as mean±SEM. Statistical comparisons between groups were made by Analysis of Variance (ANOVA). The p<0.05 was considered significant. Test of multiple comparison of Tukey was applied (5%) for statistically significant differences. The software SPSS 11.5 and Excel 2003 were used for statistical analysis and graph making, respectively.

RESULTS

Profile of VEGF production in PMA-stimulated human PBMCs:

Human PBMCs produced a noticeable amount of VEGF without any stimulus after 48 h incubation and PMA significantly reduced VEGF production by human PBMCs dose-dependently as was shown in Fig. 1 (p<0.05).

The human peripheral blood mononuclear cells (1×10⁶ cells mL⁻¹) were cultured in complete RPMI-1640 medium and then were stimulated with different concentrations of Phorbol Myristate Acetate (PMA) (0-25 ng mL⁻¹) for 48 h. At the end of incubation, VEGF concentration in conditioned medium was measured by ELISA. Data are mean±SEM of three independent experiments. *p<0.05 was considered significant.

Profile of VEGF production in PHA- stimulated human PBMCs:

Human PBMCs cultured without any stimulus, produced detectable amount of VEGF after 48 h incubation. PHA significantly increased VEGF production in human PBMCs dose-dependently as was shown in Fig. 2 (p<0.05).

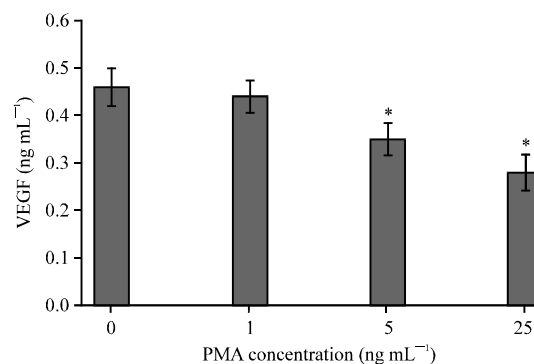


Fig. 1: Effect of PMA on VEGF production by human peripheral blood mononuclear cells

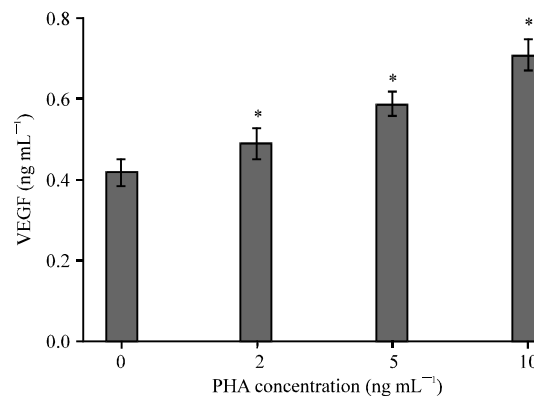


Fig. 2: Effect of PHA on VEGF production by human peripheral blood mononuclear cells

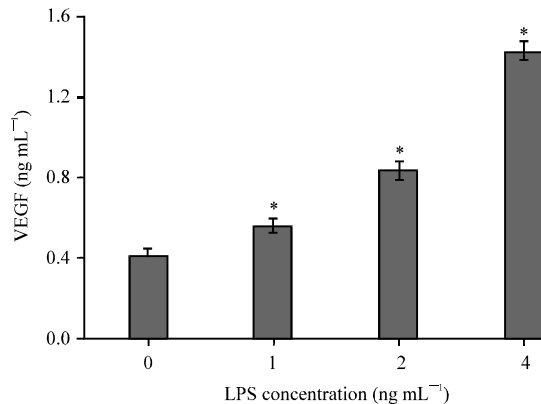


Fig. 3: Effect of LPS on VEGF production by human peripheral blood mononuclear cells

The human peripheral blood mononuclear cells (1×10^6 cells mL⁻¹) were cultured in complete RPMI-1640 medium and then were stimulated with different concentrations of Phytohemagglutinin (PHA) ($0-10 \mu\text{g mL}^{-1}$) for 48 h. At the end of incubation, VEGF concentration in conditioned medium was quantified by ELISA. Data are mean $\pm$ SEM of three independent experiments. * $p < 0.05$ was considered significant.

Profile of VEGF production in LPS-stimulated human PBMCs: Human PBMCs cultured alone (without any inducer), produced evident amount of VEGF after 48 h incubation. LPS significantly increased VEGF production in human PBMCs dose-dependently as was shown in Fig. 3 ($p < 0.05$).

The human peripheral blood mononuclear cells (1×10^6 cells mL⁻¹) were cultured in complete RPMI-1640 medium and then were stimulated with different concentrations of Lypopolysaccharide (LPS) ($0-4 \mu\text{g mL}^{-1}$) for 48 h. At the end of incubation, VEGF concentration in conditioned medium was quantified by ELISA. Data are mean $\pm$ SEM of three independent experiments. * $p < 0.05$ was considered significant.

DISCUSSION

In this study, researchers found out that human PBMCs could potentially produce VEGF. Researchers, also found that PHA/LPS increase whereas PMA decreases VEGF production in PBMCs. Different patterns of VEGF are expressed in different normal and cancer cells (Kaczmarek *et al.*, 2011). In this study, researchers used PMA, PHA and LPS as inducers of VEGF expression.

Converse to the results, induction or increase of VEGF expression in PMA-stimulated cancer and normal

cells have been shown by a number of studies. By Young, PMA increased the VEGF expression and secretion by retinal pigment epithelial cells in high glucose and hypoxia. The discrepancy between the results and Young may be in part due to the origin of cells and condition of the study. Young used retinal pigment epithelial cells in high glucose and hypoxia while researchers used PBMCs in normal condition. Also by Karroum, PMA induced VEGF expression in MCF-7 breast cancer cells. Once again the inconsistency between the results and Karroum may be somewhat due to the origin of cells. Karroum used breast cancer cells while researchers used normal PBMCs.

Moreover, researchers have shown the inhibitory effect of PMA on Matrix Metalloproteinase-9 (MMP-9) production in human PBMCs in previous study (Hajighasemi and Hajighasemi, 2011). As both of VEGF and MMP-9 have important role in angiogenesis (Tang *et al.*, 2013), it seems that the decrease of VEGF production in PBMCs by PMA may have some relation to PMA-mediated suppression of MMP-9.

Similar to the findings, induction or increase of VEGF expression in a number of cells by LPS has been reported (Lin *et al.*, 2006; Kim *et al.*, 2008; Bassols *et al.*, 2009; Van Dooren *et al.*, 2013). In Kim *et al.* (2008), LPS increased VEGF mRNA expression in rat lung pericytes dose-dependently. In Van Dooren *et al.* (2013), whole blood, PBMCs and polymorphonuclear cells were stimulated with LPS and then the cytokine secretion was determined. In Van Dooren *et al.* (2013), VEGF only was produced by PBMCs. In the present study, LPS enhanced the VEGF production by PBMCs dose dependently. Taken together Lps-stimulated PBMCs are good tools for studies on VEGF inducers/regulators.

Additionally in the study, PHA increased VEGF production in PBMCs dose-dependently. The elevated expression of VEGF in PHA-activated T cells by human dental pulp-derived stem cells has been shown (Demircan *et al.*, 2011).

Besides the role of mitogen-activated protein kinases, as key mediators of VEGF production has been shown (Kim *et al.*, 2011). Also, intervention with mitogenic signal transduction pathways reduced the expression of VEGF (Rizzo *et al.*, 2012).

The results show that PMA, PHA and LPS have different effect on VEGF production by PBMCs. PMA decreased whereas PHA/LPS increased the VEGF production in PBMCs. Moreover, it seems that LPS is a more potent inducer of VEGF production by PBMCs than PHA. Because for example as it can be seen in Fig. 2 and 3, the amount of VEGF production at $2 \mu\text{g mL}^{-1}$ of LPS is about 1.7 fold of the same dose of PHA.

PBMCs and VEGF are key regulators of inflammation (Downer *et al.*, 2013; Santer *et al.*, 2012; Yuan *et al.*, 2013;

Shaik-Dasthagirisahab *et al.*, 2013). Also, it was reported that VEGF release in mononuclear cells is of particular importance as is useful in prognosis of autoimmune disease (Correale *et al.*, 2012). Accordingly, an association between the increase in risk of Multiple Sclerosis (MS) exacerbation and enhanced disease activity with VEGF production by CD4+T cells has been revealed (Correale *et al.*, 2012). In addition, high VEGF levels in PBMCs and pulmonary edema fluid of acute hantavirus pulmonary syndrome patients has been detected (Gavrilovskaya *et al.*, 2012).

Targeting the VEGF may be precious in control of inflammation, autoimmunity and angiogenesis (Szekanecz *et al.*, 2009).

The therapeutic effect of some anti-inflammatory agents has in part attributed to their suppressive effect on VEGF secretion (Palmieri *et al.*, 2011; Gallelli *et al.*, 2013). Furthermore, the preventive effect of a number VEGF-inhibitors in autoimmunity has been shown (Seabrook *et al.*, 2010).

Important role of PBMCs and also VEGF in a number of physiologic and pathologic conditions, such as angiogenesis (neovascularization), inflammation and autoimmunity states (Sendergaard *et al.*, 2013; Sohn *et al.*, 2013) has been reported. Different profile of VEGF production by different stimulators makes PBMCs valuable screening tools for angiogenesis inducers/suppressors, VEGF enhancers/inhibitors and also study of regulatory mechanisms of VEGF secretion. Furthermore, PMA, PHA or LPs-stimulated PBMCs may be good models for study and planning the useful therapeutic approaches for inflammatory mediated diseases, selection the ideal drugs with modulatory effects on VEGF and designing of novel regulators of VEGF response in related disorders.

CONCLUSION

It seems that PBMCs provide valuable screening tools for VEGF enhancers/inhibitors and also study the regulatory mechanisms of VEGF secretion. Furthermore, PMA, PHA or LPS-stimulated PBMCs may be good models for study and planning the useful therapeutic approaches for inflammatory mediated diseases, such as autoimmunity.

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