

Lipopolysaccharide Effect on Vascular Endothelial Factor and Matrix Metalloproteinases in Leukemic Cell Lines *In vitro*

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Background: Angiogenesis, the process of new vessels generation, plays a critical role in tumor invasion and metastasis. Vascular Endothelial Growth Factor (VEGF), as a cytokine, and Matrix Metalloproteinases (MMPs), has been the important factors that involved in angiogenesis. Lipopolysaccharide (LPS) has an essential effect on angiogenesis.

Objectives: In this study the effect of LPS on VEGF production and MMP-2/MMP-9 activity in two leukemic cell lines has been assessed *in vitro*.

Materials and Methods: Human leukemic U937 and THP1 cells were cultured in complete RPMI medium. Then the cells at the exponential growth phase were incubated with different concentrations of LPS (0 - 4 µg/mL) for 48 hours. Then the level of VEGF production and MMP-2/MMP-9 activity in cell culture supernatants were evaluated with the ELISA standard kits and gelatin zymography respectively.

Results: U937 cells have produced a large amount of VEGF without any stimulus and LPS has not shown any substantial effect on VEGF production by these cells. However THP1 cells have produced a small amount of VEGF without stimulation and LPS significantly has increased VEGF production in these cells dose-dependently. Moreover LPS significantly has augmented the MMP-2/MMP-9 activity in the both leukemic cell lines in a dose-dependent manner.

Conclusions: Our results have shown that LPS might be a potential inducer/enhancer of VEGF production and MMP-2/MMP-9 activity (angiogenic factors) in leukemia. Moreover the LPS effect on angiogenesis might be in part, due to its stimulatory effects on VEGF and MMPs. Overall LPS-stimulated leukemic cells might be good models for study and planning the useful therapeutic approaches for angiogenesis-dependent diseases.

Keywords: VEGF; MMP; LPS; Leukemia; Angiogenesis

1. Background

Angiogenesis (creation of new vessels or replenish of the pre-existing vascular networks) plays a critical role in some pathological conditions such as tumor expansion and metastasis (1-3). Many factors have involved in angiogenesis including hormones, cytokines and Matrix Metalloproteinases (MMPs) (4-6). Vascular Endothelial Growth Factor (VEGF) is a cytokine which is produced by a number of cells and has a key role in angiogenesis and inflammation (5, 7). VEGF is overexpressed in many malignant cancers (8, 9). The increased VEGF level has been reported in serum of leukemia patients and also leukemia cell culture supernatants (8, 10). MMPs were a large group of enzymes that have break down extracellular matrix result in tissue remodeling and have an important role in angiogenesis and inflammation (6, 11). Lipopolysaccharide (LPS) is endotoxin constitute the major component of outer membrane in gram negative bacteria (12). The induction of angiogenesis by LPS has been shown (13-15). Also LPS has increased the VEGF production in peripheral blood mononuclear cells *in vitro* (16). Moreover the

effects of LPS in leukemiogenesis and *in vitro* activation of leukemic cells have been reported (17, 18). Besides the angiogenesis and angiogenic factors have important role in leukemia (19, 20).

2. Objectives

In this study the effect of LPS on angiogenic factors in two leukemic cell lines has been studied *in vitro*.

3. Materials and Methods

3.1. Reagents

RPMI-1640 medium, penicillin, streptomycin, Lipopolysaccharide (LPS), 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 has obtained from R and D company (USA).

3.2. Cell Lines

Human leukemic monocytes [U937 (NCBI C130) and THP1 (NCBI C563)], have obtained from NCBI (National Cell Bank of Iran, Pasteur Institute, Tehran, Iran). The cells have maintained in RPMI-1640 medium supplemented with 10% FCS and incubated in 5% CO₂ at 37°C.

3.3. Cell Culture and Treatment

The method has described in detail elsewhere (10). Briefly, the human leukemic cells were cultured in RPMI-1640 medium supplemented with 10% FCS, penicillin (100 IU/mL) and streptomycin (100 µg/mL) at 37°C in 5% CO₂. The cells were seeded at a density of 106 cells/mL and prior to experiments were treated in fresh medium. Then the cells were incubated with different concentrations of LPS (0 - 4 µg/mL) for 48 hours. The supernatants of cell cultures were collected, centrifuged and stored at -80°C for next experiments. All experiments were done in triplicate.

3.4. Evaluation of VEGF Production by ELISA

The amount of VEGF have secreted in the cell culture supernatants by human leukemic cell lines 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.

3.5. Evaluation of MMP-2/MMP-9 Activity by Gelatin Zymography

MMP-2/MMP-9 activity in cell-conditioned media was evaluated by gelatin zymography technique according to the modified Kleiner and Stetler-Stevenson method (21). Briefly cell culture supernatants were subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) on 10% polyacrylamide gel copolymerized with 2 mg/mL gelatin A in the presence of 0.1% SDS under non-reducing conditions at a constant voltage of 80 V. After electrophoresis, gels were washed in 2.5% Triton X-100 for one hour to remove SDS and then incubated in a buffer containing 0.1 M Tris-HCl, PH 7.4 and 10 mM CaCl₂ overnight at 37°C. Afterwards the gels were stained with 0.5% Coomassie Brilliant Blue and then destained. Proteolysis activities of enzyme were detected as clear bands of gelatin lysis against a blue background. The relative intensity of lysed bands to control were measured by using UVI Pro gel documentation system (GDS_8000 system) and expressed as relative gelatin lytic activity.

3.6. Statistical Analysis

VEGF level quantification and relative gelatinolytic activity of MMP-2/MMP-9 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). 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 used for statistical analysis and graph making respectively.

4. Results

4.1. Effect of LPS on VEGF Production in Human Leukemic Cell Lines

Results depicted in Figure 1, demonstrate that U937 cells produced a large amount of VEGF without any stimulation and LPS did not show any significant effect on VEGF production by these cells.

Furthermore according to our findings, VEGF production was rather low in unstimulated THP1 cells but LPS significantly increased VEGF production in these cells after 48 hours incubation time dose dependently as can be seen in Figure 1 (P < 0.05).

4.2. Effect of LPS on MMP Activity in Human Leukemic Cell Lines

4.2.1. Effect of LPS on MMP-2 Activity in Human Leukemic Cell Lines

When U937 cells cultured without any stimulation, faint bands related to MMP-2 activity were detectable and LPS significantly increased MMP-2 activity in U937 cells after 48 hours incubation time in comparison with untreated control cells. The enhancing effect of LPS on MMP-2 activity in U937 cells was dose-dependent as has depicted in Table 2 and Figure 2 (P < 0.05).

However MMP-2 activity in unstimulated THP-1 leukemic cells was not detectable and LPS induced MMP-2 activity in these cells after 48 hours incubation time dose dependently compared with untreated control cells as it has shown in Table 2 and Figure 2 (P < 0.05).

4.2.2. Effect of LPS on MMP-9 Activity in Human Leukemic Cell Lines

When U937 cells cultured alone with no stimulus, faint bands related to MMP-9 activity have detected. LPS stimulation, significantly increased MMP-9 activity in U937 cells compared with untreated control cells. The LPS increased MMP-9 activity in U937 cells was dose-dependent as illustrated in Table 3 and Figure 3 (P < 0.05).

Nevertheless MMP-9 activity in unstimulated THP-1 leukemic cells was not evident and LPS induced MMP-9 activity in these cells after 48 hours incubation time dose dependently compared with untreated control cells as it has shown in Table 3 and Figure 3 (P < 0.05).

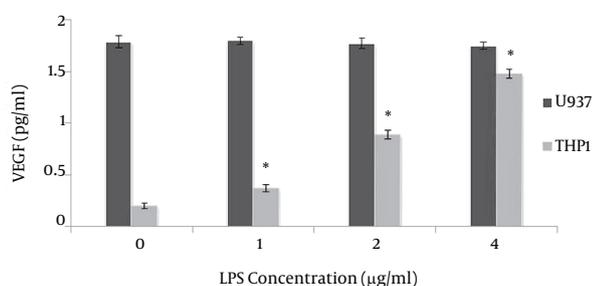
Table 1. Effect of LPS on VEGF Production in Human Leukemic Cell Lines ^{a,b}

Cell Lines	LPS Concentratio, µg/mL		
	1	2	4
U937	1.8 ± 0.035, 0.7	1.77 ± 0.68, 0.05	1.75 ± 0.04, 0.4
THP1	0.37 ± 0.035, 0.0034	0.89 ± 0.04, 0.000032	1.48 ± 0.04, 0.0000024

^a P < 0.05 was considered significant.

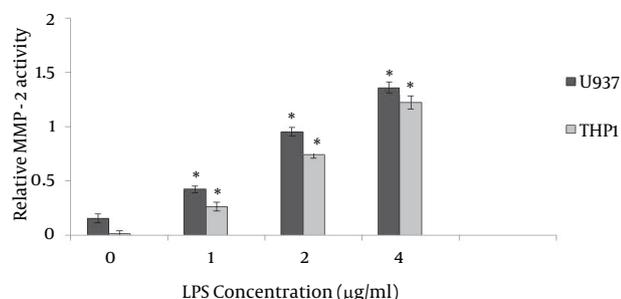
^b Values are presented as Mean ± Standard Deviation, PV.

Figure 1. Effect of LPS on VEGF Production by U937 AND THP1 Leukemic Cells



The leukemic cells (1×10^6 cells/mL) were cultured in complete RPMI-1640 medium and then were stimulated with different concentrations of lypopolysaccharide (LPS) (0 - 4 µg/mL) for 48 hours. At the end of incubation, VEGF concentration in conditioned medium was quantified by ELISA. Data are Mean ± SEM of three independent experiments. *P < 0.05 was considered significant.

Figure 2. Effect of LPS on MMP-2 Activity in U937 and THP1 Leukemic Cells



The U937 leukemic cells (1×10^6 cells/mL) were cultured in complete RPMI-1640 medium and then were stimulated with different concentrations of lypopolysaccharide (LPS) (0 - 4 µg/mL) for 48 hours. At the end of incubation, the MMP-activity in conditioned medium was quantified by gelatin zymography. Data are Mean ± SEM of three independent experiments. *P < 0.05 was considered significant.

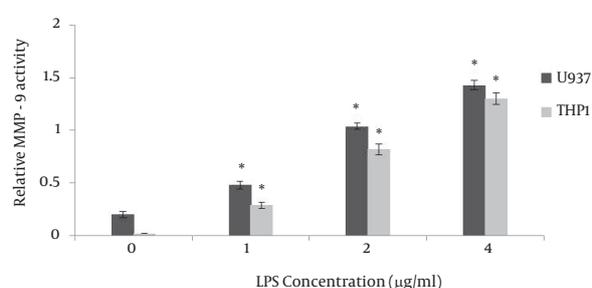
Table 2. Effect of LPS on MMP-2 Activity in Human Leukemic Cell Lines ^{a,b}

Cell Lines	LPS Concentratio, µg/mL		
	1	2	4
U937	0.42 ± 0.032, 0.00094	0.95 ± 0.04, 0.000017	1.36 ± 0.05, 0.000016
THP1	0.26 ± 0.04, 0.00534	0.74 ± 0.035, 0.00772	1.22 ± 0.06, 0.00046

^a P < 0.05 was considered significant.

^b Values are presented as Mean ± Standard Deviation, PV.

Figure 3. Effect of LPS on MMP-9 Activity in U937 and THP1 Leukemic Cells



The THP1 leukemic cells (1×10^6 cells/mL) were cultured in complete RPMI-1640 medium and then were stimulated with different concentrations of lypopolysaccharide (LPS) (0 - 4 µg/mL) for 48 hours. At the end of treatment, MMP-9 activity in conditioned medium was measured by gelatin zymography. Data are Mean ± SEM of three independent experiments. *P < 0.05 was considered significant.

Table 3. Effect of LPS on MMP-9 Activity in Human Leukemic Cell Lines ^{a,b}

Cell Lines	LPS Concentratio, µg/mL		
	1	2	4
U937	0.48 ± 0.04, 0.0011	1.36 ± 0.03, 0.0000051	1.43 ± 0.045, 0.0000096
THP1	0.29 ± 0.03, 0.0039	0.82 ± 0.052, 0.0013	1.3 ± 0.056, 0.0006

^a P < 0.05 was considered significant.

^b Values are presented as Mean ± Standard Deviation, PV.

5. Discussion

The results of this study have shown that U937 and THP1 leukemic cells were potential producers of VEGF, MMP-2 and MMP-9. Accordingly, U937 cells have produced a

great amount of VEGF without any stimulus and LPS has not shown any significant effect on VEGF production by U937 cells. Whereas THP1 cells have produced an evident amount of VEGF without stimulation and LPS has significantly increased VEGF secretion in this cell line. Therefore our results have shown that there are various patterns of VEGF production in leukemic cells and LPS had different

effect on VEGF production in different leukemic cells. Consequently sensitivity of various leukemic cells to LPS-induced VEGF secretion is different. Diverse patterns of VEGF expression in tumorous and normal tissues have been revealed and different cancers exhibit different profiles of VEGF secretion (22-24). VEGF has a key role in tumor expansion and metastasis (25, 26) and is an important biomarker of tumor (27). Thus VEGF might be a good prognostic and diagnostic marker (28-31). Similar to our findings, the presence of VEGF-C protein in supernatants of PMA-differentiated HL-60 leukemic cells after LPS stimulation has shown (32). Also increase of VEGF production by LPS in peripheral blood mononuclear cells has been revealed *in vitro* (16). Moreover LPS treatment of murine skin wounds with topical LPS has increased the secretion of some growth factors including VEGF at the wound site (33). Furthermore induction of angiogenesis by LPS has been shown (13-15). In addition VEGF has a key role in angiogenesis (5, 7). So the induction of angiogenesis by LPS might be in part due to its stimulatory effect on VEGF production. Currently the anti-leukemic effects of VEGF inhibitors has been reported (34, 35). So inhibition of LPS might have potential implication in treatment of leukemia.

According to the results of this study, LPS increased MMP-2/MMP-9 activity in U937 leukemic cells, and induced MMP-2/MMP-9 in THP1 leukemic cells. Consequently pattern of MMP-activity in U937 and THP1 cells was somewhat different. Similar to our findings, diverse profiles of MMPs expression in various tumor cells have been reported (36, 37). MMPs had an important role in angiogenesis (6, 11). The induction of angiogenesis by LPS has been also shown (13-15). Thus the stimulation of angiogenesis by LPS might be partially due to its stimulatory effect on MMP-2/MMP-9 activities. In addition the effects of LPS in leukemogenesis and *in vitro* activation of leukemic cells have been reported (17, 18). Therefore inhibition of LPS-induced MMP activity might be a novel possible approach in leukemia therapy. Furthermore preclusion of apoptosis in neutrophils by LPS has been shown (38). Also production of Tumor necrosis factor- α (TNF- α) by LPS-stimulated THP-1 cells has been revealed (39). Most important topic would be that LPS had an essential role in induction of tumor metastasis (40). Both VEGF and MMPs had important role in cancer metastasis (41). Increase of VEGF expression by LPS in several cells including rat lung pericytes and PBMCs has been shown (16, 42, 43). So the role of LPS in metas-

tasis might partly due to its stimulatory effect on VEGF production and MMP-2/MMP-9 activities. The correlation between angiogenesis and development of leukemia particularly CLL has been reported by several studies (44-46). The induction of angiogenesis by LPS has been also shown (13-15). Thus LPS might have noticeable effect in leukemia progression through stimulation of pathologic angiogenesis.

As a whole avoidance/treatment of bacterial infections especially their LPS could be essential in management of tumors such as leukemia in which pathologic angiogenesis has played considerable role. In addition according to the results of this study, LPS-stimulated U937 and THP1 leukemic cells could be precious screening tools for VEGF/MMP inhibitors and subsequently planning the novel drugs for treatment of refractory leukemias. Also other studies about LPS effects on VEGF/ MMP expression in other malignant cells as well as *in vivo* models have been warranted.

LPS-stimulated U937 and THP1 leukemic cells could be useful tools for screening of VEGF/MMPs modulators and therefore setting up of novel drugs for treatment of leukemia and other related cancers.

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Authors' Contributions

Fatemeh Hajjighasemi, designed and performed the study, collected and analyzed the data and wrote this article. Mohmmad Hossein Gheini, revised the paper. All authors read and approved the final manuscript.

Conflict of Interest

The authors made no disclosures.

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