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The Increase of pFAK and THBS1 Protein and Gene Expression Levels in Vascular Smooth Muscle Cells by Histamine-treated M1 Macrophages

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ABSTRACT

Atherosclerosis is developed due to the formation of atheroma plaques in the coronary arteries. In this process, M1 macrophages and vascular smooth muscle cells (VSMCs) are the main functional cells. Inflammatory mediators such as histamine may inflame M1 macrophages. The aim of this study was to determine the effect of M1 macrophage secretion contents on the gene and protein expression levels of focal adhesion kinase (FAK), vasodilator-stimulated phosphoprotein (VASP), and thrombospondin1 (THBS1).

Whole blood samples from the six healthy subjects (stenosis<5%), and six patients (stenosis>70%) were prepared and peripheral blood mononuclear cells (PBMCs) were isolated. Then monocytes were differentiated into M1 macrophages using 100 ng/mL granulocyte-macrophage colony stimulating factor (GM-CSF). The differentiated M1 macrophages were treated with histamine (10^{-6} M), and their secretion contents were harvested and added to the culture medium of VSMCs. The FAK, VASP, and THBS1 gene expression and protein levels were measured using RT-qPCR and western blot techniques in VSMCs, respectively.

The FAK and THBS1 gene expression levels significantly increased in VSMCs after adding secretion contents obtained from histamine-treated M1 macrophages (p=0.023 and 0.05, respectively), while significant results were not observed for VASP gene (p=0.45). In converse with the phosphorylated VASP (pVASP) (p<0.34), the phosphorylated FAK (pFAK) and THBS1 protein levels increased in VSMCs (p<0.001).

We concluded that in inflammatory conditions, the immune events could affect the macrophages by histamine. The activated macrophages could locally activate signaling

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pathways via FAK and THBS1 genes that are effective in the proliferation and migration of VSMCs.

Keywords: Atherosclerosis; Focal adhesion kinase; Histamine; Macrophages; Thrombospondin 1; Vascular smooth muscle cells; Vasodilator-stimulated phosphoprotein

INTRODUCTION

Atherosclerosis is known as one of the most common problems of human societies in both developed and developing countries. The progression of atherosclerosis in coronary arteries may lead to coronary artery diseases, and myocardial infarction.[1] Atherosclerosis develops due to hardening of the vessel wall by the formation of atheroma plaques in the lumen of arteries.[2] The inflammation events are reported the main cause of plaques formation within the vessel wall.[3] The plaque remodeling occurs because of the function of inflammatory cells such as monocytes and M1 macrophages in the sub-endothelial space, and the consequent activation of vascular smooth muscle cells (VSMCs).[4] The M1 macrophages are involved in many biological processes and the pathogenesis of other diseases. The circulating monocytes enter into the sub-endothelial space and differentiate into M1 macrophages.[5] The various agents can influence the activation of macrophages. Histamine, known as an inflammatory mediator, plays an important role in the function of the cardiovascular system.[6] The histamine is produced from L-histidine by histidine decarboxylase and acts via the H1 receptor.[7] It has an immunoregulatory function on immune cells and plays an important role in the early stages of inflammatory events.[8] In stress conditions, the production of histamine increases and intensifies the process of inflammation. [9]

VSMCs are another major cell of the coronary artery wall and migrate to the inflammatory area during the early stages of atherosclerosis.[10,4] In the process of activation and proliferation of VSMCs, many genes are involved. Some signaling pathways are activated by integrin receptors on the VSMCs surface. Focal adhesion kinase (FAK), also called protein tyrosine kinase 2 (PTK2), as cytosolic protein plays a very important role in the early stages of cell migration, and focal adhesion. FAK is phosphorylated in response to stimulation of the integrin. Thrombospondin1 (THBS1) as a secreted matricellular and homotrimeric glycoprotein plays important roles in cell proliferation, migration, and focal adhesion. As an adhesive protein, it is upregulated in vessel wall cells after vascular injury and is activated through integrins.[11,12] Vasodilator-stimulated phosphoprotein (VASP), as an intracellular protein, may participate in signaling pathways and regulate cell-to-cell responses. It may play a role in focal adhesion and cell motility.[13]

In this study, monocytes were isolated from the whole blood of patients with the stenosis of coronary arteries and healthy subjects and were differentiated into M1 macrophages. Then, the secretion contents of histamine-stimulated M1 macrophages were applied to evaluate the FAK, VASP, and THBS1 gene and protein expression changes in the VSMCs.

MATERIALS AND METHODS

Sample

The whole blood samples were prepared from twelve subjects (12 men, 60-70 years old) undergoing the coronary artery angiography in Tehran Hazrat Rasool general hospital. They have contained six healthy subjects (stenosis <5%), and six patients (with stenosis >70% in main vessels) following the current drug regimes. Not all volunteers had other disorders, including metabolic and inflammatory diseases. A written consent form was obtained from all subjects. The study was approved by the Ethics Committee of the Iran University of Medical Sciences (N.: IR.IUMS.REC.1395.9221184205).

PBMC Isolation

10 mL of whole blood samples were collected in EDTA-containing tubes and were transferred to the lab at 4°C. The peripheral blood mononuclear cell (PBMC) samples were isolated using a combinational technique containing ficoll (GE Healthcare Life Sciences, CAT.#:17-1440-02) and RosetteSep kit

(Human Monocyte Enrichment Cocktail, Stemcell Technologies, Singapore, CAT.#:15068), based on an immunodensity protocol. The RosetteSep isolate monocytes from blood by negative selection. Other cells especially red blood cell (RBC) are targeted and removed by tetrameric antibody complexes. At first, the whole blood was diluted with phosphate-buffered saline (PBS) buffer (pH 7.4) as 1:1 ratio v/v. Then, it was added to the ficoll solution (3 mL) containing the RosetteSep cocktail (150 µL) and was centrifuged (20 min, 25^0C, at 1200 g). Finally, the PBMC layer was harvested and washed three times with PBS.

Monocyte Differentiation

The PBMCs were cultured in RPMI1640 (Gibco, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, NY, USA). They were differentiated into M1 macrophages using 100 ng/mL granulocyte-macrophage colony stimulating factor (GM-CSF) (Cell Signaling Technology, Beverly, MA, USA, CAT.#:8922) at 7 days. Finally, the differentiated macrophages were treated with histamine (Sigam-Aldrich, Switzerland, CAT.#:59964) (10^{-6} M, 48 hours) and their culture mediums were harvested (with histamine). Then CD68 and CD80 markers were studied by flow cytometry technique.

Cell Culture

Vascular smooth muscle cells (VSMCs) were purchased from the National Cell Bank, Pasteur Institute of Iran (NCBI Code, C591). They were cultured in DMEM-F12 (Gibco, NY, USA), supplemented with 0.01 mg/mL insulin, 0.03 mg/mL bFGF, 0.1 mg/mL EGF, containing 80% secretion contents of M1 macrophages.

Gene Expression

Total RNA was extracted from VSMCs by Bio basic Kit, (Canada, CAT.#:BS1361). cDNA synthesis was performed according to the Takara Kit (Prime Script II strand cDNA Synthesis Kit, Takara, Japan, CAT.#:6110A). The gene expression levels of focal adhesion kinase (FAK), vasodilator-stimulated phosphoprotein (VASP), and thrombospondin1 (THBS1) were measured by Takara Kit (Takara, Japan, CAT.#RR420A), using SYBER Green RT-qPCR technique (Applied Biosystems). The gene expression level was normalized by the beta-actin gene. The reactions were performed for all genes in a volume of 15 µl and 45 cycles. The temperature cycle was performed at 95^0 (10 s) and 64ºC (30 s). Primers were designed using the Primer-BLAST (NCBI.nlm.nih.gov/tools/primer-blast), GeneRunner (Version 6.5.51), and OligoCalc (Version 3.27) tools (Table 1). The gene expression changes were identified with $2^{-\Delta\Delta CT}$ formula.

Western Blotting

VSMCs were cultured in DMEM-F12, supplemented with the culture contents of M1 macrophages for two days. Then, they were lysed in RIPA Lysis Buffer (pH 7.2, NaCl 0.15 M, HEPES 50 mM, EDTA 2 mM, NP-40 0.1% and sodium deoxycholate 0.05%), and were centrifuged at 13,000 g (4^0C, 20 minutes). Proteins concentration was identified by BCA (bicinchoninic acid assay) protein quantification Kit (Parstous, Iran, CAT.#:A101251). The supernatants were mixed with sample buffer and were separated using SDS-PAGE (10%). Then, the separated protein bounds were transferred to PVDF membrane (Immobilon-P, Germany, CAT.#:IPVH00010). After blocking step at 3 hours with 5% skim milk (Cell Signaling Technology, Beverly, MA, USA, CAT.#:9999), PVDF membrane was washed 3 times, and incubated with phosphorylated FAK (pFAK) (Tyr397) Antibody (Cell Signaling Technology, Beverly, MA, USA, CAT.#:3283s), phosphorylated VASP (pVASP) (Ser157) Antibody (Cell Signaling Technology, Beverly, MA, USA, CAT.#:3111s), and THBS1 Antibody (Cell Signaling Technology, Beverly, MA, USA, CAT.#:14778s), overnight at 4°C. PVDF membrane was incubated (3 hours, on the stirrer at room temperature) with Anti-Rabbit IgG, HRP-linked Antibody (secondary antibody) (Cell Signaling Technology, Beverly, MA, USA, CAT.#:7074s). Finally, proteins were visualized with the ECL reagents (Amersham Biosciences, Italy, RPN2235), exposed to X-ray film, and its density was quantified by ImageJ software. Beta-actin (Cell Signaling Technology, Beverly, MA, USA, CAT.#:4967s) was used to normalize the protein values.

Protein Prediction

Based on bioinformatics studies, a large number of genes involved in atherosclerosis pathophysiology and the activation of VSMCs were identified by high-throughput studies.[14] The genes proposed in the

activation, proliferation, and migration of VSMCs were identified by the data banks of the STRING version 10.5 (String-db.org) and KEGG (Genome.jp/kegg). Eventually, three genes in one signaling pathway with high scores including FAK, VASP, and THBS1 were selected.

Statistics

The data was analyzed by IBM SPSS Statistics, version 24 (IBM Corp., Armonk, N.Y., USA). Initially, data distribution was evaluated by the Kolmogorov-Simonov test. The $p<0.05$ was the subject to consider significant. The gene expression levels changes were identified with $2^{-\Delta\Delta CT}$ formula.

Table 1. Forward and reverse primers for focal adhesion kinase (FAK), thrombospondin 1 (THBS1), vasodilator-stimulated phosphoprotein (VASP) and beta-actin genes

Gene	Forward primer	Reverse primer
Beta-Actin	TCCCTGGAGAAGAGCTACG	GTAGTTTCGTGGATGCCACA
FAK	CATGCCCTCAACCAGGGATT	CACGCTGTCCGAAGTACAGT
VASP	CAGGAAAGTCAGCAAGCAGG	TGGCAGATTCATCCTTGGGG
THBS1	AGGACTGCGTTGGTGATGTA	TCAGGCACTTCTTTGCACTCAT

RESULTS

Differentiation of Monocytes into M1 Macrophages

Monocytes cultured and differentiated into M1 macrophages using granulocyte-macrophage colony stimulating factor (GM-CSF) after seven days. After the intervention with histamine, the flow cytometry results confirmed the CD68 expression level (known as positive CD marker for M1 macrophages) up to 87.25% in the macrophages. However, the CD80 expression level was not as high as observed for the CD68 (CD68+ CD80+ macrophages, 6.63%) (Figure 1).

Focal Adhesion Kinase (FAK), Vasodilator-Stimulated Phosphoprotein (VASP), and Thrombospondin1 (THBS1) Gene Expression Levels

The results showed that the secretion content of histamine-treated M1 macrophages increases the FAK gene expression level in significantly the vascular smooth muscle cells (VSMCs) as compared to control group ($p=0.023$). The similar significant result ($p=0.05$) found for THBS1 gene expression level (2.5 times). However, the VASP gene expression level in the VSMCs did not change significantly by the secretion contents obtained from histamine-treated M1 macrophages ($p=0.45$) (Figure 2).

Phosphorylated FAK (pFAK), Phosphorylated VASP (pVASP), and THBS1 Protein Levels

The secretion contents of histamine-treated M1 macrophages increased significantly the phosphorylation of the pFAK protein in the VSMCs up to three times ($p<0.001$). Furthermore, the similar results observed for the THBS1 secretion protein so that there was a significant increase 2.5 times in the VSMCs ($p<0.001$). There were no significant changes in the phosphorylation of VASP protein in VSMCs after adding the secretion contents obtained from the histamine-treated M1 macrophages ($p=0.34$) (Figure 3).

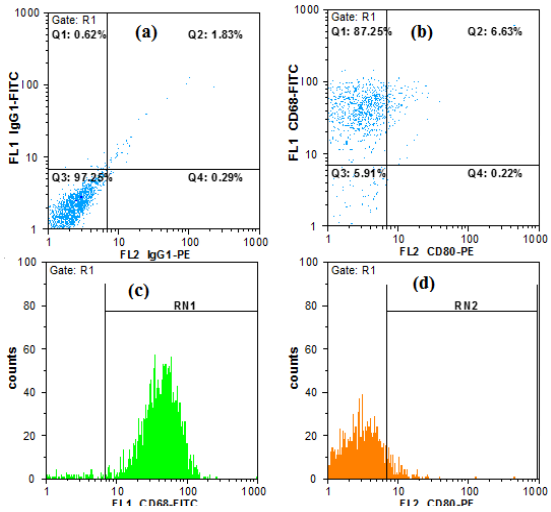

Figure 1. CD68- and CD80-Macrophage flow cytometry. (a) Unstained cell dot plot. (b) CD68-fluorescein isothiocyanate (FITC)-conjugated stained cell (87.25%), CD80-phycoerythrin (PE)-conjugated stained cell (0.22%) dot plots. (C) CD68-FITC-conjugated stained cell histogram. (D) CD80-PE-conjugated stained cell histogram.

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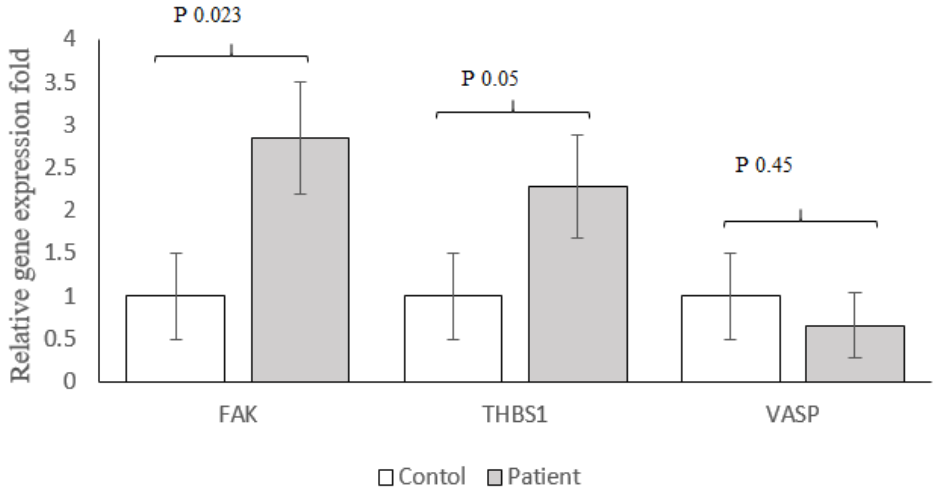

Figure 2. Focal adhesion kinase (FAK), thrombospondin 1 (THBS1), vasodilator-stimulated phosphoprotein (VASP), and beta-actin genes expression levels in vascular smooth muscle cells (VSMCs) cultured in secretion contents of M1 macrophages. The VSMCs treated with the secretion contents of differentiated M1 macrophages. The FAK and THBS1 gene expression levels increased significantly (*p*=0.023 and *p*=0.05, respectively) as compared with the VASP gene (*p*=0.45) in the VSMCs.

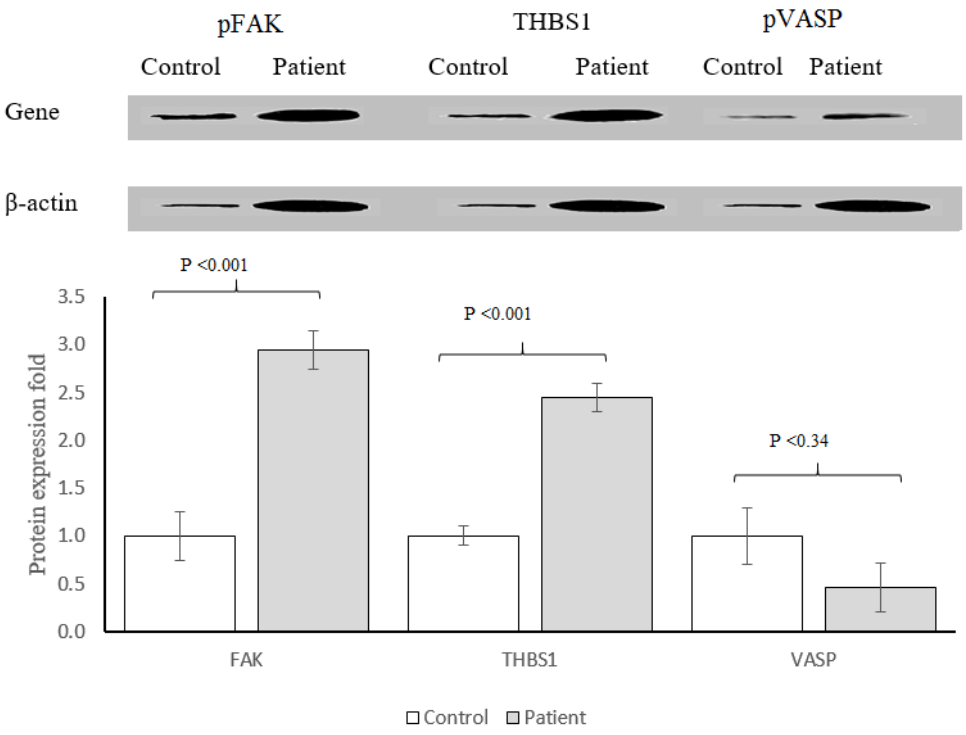

Figure 3. Phosphorylated focal adhesion kinase (pFAK), thrombospondin 1 (THBS1), phosphorylated vasodilator-stimulated phosphoprotein (pVASP), and beta-actin protein changes in vascular smooth muscle cells (VSMCs) cultured in secretion contents of M1 macrophages. The VSMCs treated with the secretion contents of differentiated M1 macrophages. In converse with VASP (*p*<0.34), pFAK form and THBS1 value increased significantly (*p*<0.001) in VSMCs.

DISCUSSION

Atherosclerosis is reported as one of the most common cardiovascular disease recognized in the world. It especially in coronary arteries, leads to lethal diseases such as myocardial infarction.[1] The main cause is the hardening of the vascular wall due to the formation of atheroma plaque.[2] Furthermore, chronic inflammatory responses of inflammatory cells such as monocytes and macrophages are involved in the activation of vascular smooth muscle cells (VSMCs).[3,4] Generally, the monocytes migrate and differentiate into macrophages in the intima.[5] The M1 macrophages have pro-inflammatory and apoptotic properties and play an important role in plaque formation.[15] The various pathologic and hormonal factors, inflammatory events and oxidative stress can affect the differentiation of monocytes into M1 macrophages. Histamine with immunoregulatory function has an important role in the initiation of inflammatory conditions in the cardiovascular tissue.[6] It has been shown that histamine stimulates the production of immunomodulatory and pro-inflammatory cytokines such as IL10, IL6, IL8, and TNF-α in arterial wall.[16-19] Histamine also causes the production of matrix metalloproteinases[7], the cell to cell adhesion, the increased expression of H1 receptor expression, and the increased proliferation of VSMCs and macrophages in the intima.[20] Therefore, in the process of atherosclerosis, histamine can increase the differentiation of monocytes into macrophages, the increase of the expression of H1 receptors on macrophages, and the increase of the stimulation of M1 macrophages by secretion content of inflammatory mediators.[6] As a result, histamine was used to stimulate M1 macrophages. Many studies reported that the M1 macrophages express the CD68 marker.[21,22] However, the induction of gene markers was different depending on the M1 stimulators. Some studies reported that the LPS, INFγ,[23] IL4,[24] IL13,[25] IL1b, and IL10[26] affect the CD80 gene expression on the M1 markers. Thus, we suggested the reduction of CD80 marker may be due to treating the granulocyte-macrophage colony stimulating factor (GM-CSF) polarized macrophages with histamine in this study.

VSMCs are one of the main cells involved in the formation of plaque atheroma (fibrofatty).[10] In arterial injury and chronic inflammation, the phenotype and function of VSMCs may be changed into proinflammatory ones. They can proliferate and migrate towards the damage region on the artery wall.[10] Thus, it can be assumed that the inflammatory secretions of macrophages may activate VSMCs.[4] Some factors including macrophage inflammatory mediators can also activate integrins on the surface of VSMCs. It has been determined that the migration of VSMCs is activated through interactions of integrins with extracellular matrix (ECM) and inflammatory mediators.[27] Many genes and regulatory molecules are known to be involved in the function of VSMCs. Focal adhesion kinase (FAK) plays a very important role in cell migration and focal adhesion and is phosphorylated in response to stimulation of the integrins. A study has shown that FAK induces the migration of VSMCs through RhoA/ROCK1 signaling pathway.[28,29] Also, treatment of VSMCs with oxidized-low density lipoprotein (ox-LDL) leads to increased proliferation and mobility by activation of integrins and FAK.[27] In this regard, our results showed that M1 macrophage secretion content increases the FAK gene expression level and its active form in the VSMCs. Thrombospondin 1 (THBS1) is involved in the cell to ECM and cell to cell interactions. It plays a role in cell proliferation, migration and focal adhesion.[30] It participates in the inflammatory process, but its role in the process of atherosclerosis is not well-known.[31] It can be said, THBS1 increased the processes of VSMCs proliferation and migration in neointima formation.[32] Also, due to its effect on increasing the proliferation of VSMCs, it has been described as a proatherogenic agent.[33] However, a study showed that defective THBS1 increases the formation of atherosclerotic plaques.[31] Furthermore, the stimulation of VSMCs migration by THBS1 leads to phosphorylation of PI3-K resulting in phosphorylation of FAK.[34,35] Under inflammatory and stimulatory conditions, the activated VSMCs release THBS1 to ECM. Apparently, it is essential for the proliferation and migration of VSMCs.[12] Similar to previous studies, our results showed that M1 macrophage secretion contents increase the THBS1 gene and protein expression levels. Vasodilator-stimulated phosphoprotein (VASP), as an intracellular protein, plays an essential role in focal adhesion and cell motility, by regulating the actin cytoskeleton.[36] However, no study has been done about its role in the processes of vascular cellular activation and complication. In converse to the THBS1 and FAK

proteins, our results showed that the secretion of macrophages does not increase the VASP expression level and its activated form as compared to the control group. The results showed that the histamine-inflamed M1 macrophage secretion contents could activate several signaling pathways involved in VSMC proliferation and migration. However, in this study, only three genes involved in proliferative signaling pathways were investigated; therefore, we suggest that the study should be continued experimentally in research fields.

We concluded that in inflammatory conditions, the immune events could affect the macrophages by histamine. The activated macrophages could locally activate signaling pathways via FAK and THBS1 genes that are effective in the proliferation and migration of VSMCs.

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