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Aspirin inhibits monocyte chemoattractant protein-1 and interleukin-8 expression in TNF-α stimulated human umbilical vein endothelial cells

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Abstract

Atherosclerosis and its complications such as stroke, myocardial infraction and peripheral vascular disease, remain the major causes of morbidity and mortality in the world. Studies have showed that chemokines and adhesion molecules are involved in causing atherosclerosis by promoting directed migration of inflammatory cells. Monocyte chemoattractant protein-1 (MCP-1) is one of the key factors critical for the initiating and developing of atherosclerotic lesions. IL-8, a CXC chemokine, stimulates neutrophil chemotaxis. Aspirin is the most common drug used to prevent the complications of atherosclerosis such as stroke and coronary heart disease. In this study, we found that aspirin inhibited TNF-α (10 ng/ml)-induced MCP-1 and IL-8 expression at the RNA and protein levels in human umbilical vein endothelial cells (HUVECs), monocyte adhesion and transmigration, and that its inhibitory effects were not due to decreased HUVEC viability as assessed by MTT test. Aspirin at the dose as low as 10 μg/ml significantly inhibited the release of TNF-stimulated MCP-1 by 29.1% ($P = 0.008$) and IL-8 by 26.9% ($P = 0.0146$) as compared to TNF-stimulated release. Antibodies pretreatment were likely to decrease the production of MCP-1 ($P < 0.0001$) and IL-8 ($P < 0.0001$). Furthermore, aspirin (10 μg/ml) inhibited U937 cell adhesion by a 13.4% ($P = 0.0119$) inhibition as compared to TNF-stimulated alone. Finally, at higher concentration, aspirin also inhibited U937 migration to HUVEC by 89.1% ($P = 0.0475$) as compared to TNF-stimulated alone. These results in our study suggest that aspirin inhibits TNF-α stimulated MCP-1 and IL-8 release in HUVECs, for its additional therapeutic effects of aspirin in causing atherosclerosis.
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Keywords: Monocyte chemoattractant protein-1; IL-8; Human vascular endothelial cell; Atherosclerosis; Aspirin

1. Introduction

Chemokines, a family of low-molecular-weight proteins (8–12 kDa), which can cause the directed migration of leukocytes in vitro, are produced by endothelial cells (ECs) in response to molecules involved in inflammatory reactions, immunity and thrombosis [1,2]. The EC chemokine includes members of both CXC (IL-8, epithelial-cell-derived neutrophil-activating protein 78 [ENA-78], growth-related oncogene α [GRO-α], and interferon-inducible protein 10 [IP-10]) and CC (monocyte chemoattractant protein [MCP-1], MCP-3, and RANTES) [3]. Monocyte adhesion, migration, and infiltration into the atheroma are crucial to cause atherosclerosis. Chemokines mediate, in part, in recruiting monocytes and lymphocytes into sites of atherosclerotic lesions [1,2,4].

Monocytes can be selectively attracted to a specific chemokine, MCP-1, which is a 14-kDa glycoprotein of the CC chemokine family and a potent chemoattractant for monocyte recruitment [5–7]. MCP-1 is expressed by various cell types including monocytes, smooth muscle cells, and human vascular endothelial cells (HUVECs) in response to

Abbreviations: MCP-1, monocyte chemoattractant protein-1; HUVEC, human umbilical vein endothelial cells; TNF, tumor necrosis factor; IL-8, interleukin-8

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several different stimuli such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IgG [8–10]. MCP-1 has been found as one of the key factors to start the inflammatory process in atherogenesis [1,5,11]. Instead of RANTES, MCP-1 has detected in macrophage-rich areas of atherosclerotic lesions with an anti-MCP-1 antibody [12] and in situ hybridization [13]. MCP-1 mRNA expression has been detected in endothelial cells, macrophages and vascular smooth muscles cells in atherosclerotic arteries in surgical patients receiving bypass revascularization [14]. Therefore, MCP-1 is critical to initiate and develop atherosclerotic lesions. Furthermore, IL-8, another important chemokine found in human atheroma [18], has been found act mainly on neutrophils by most investigators [15,16] but also recruit monocytes in some studies [2,17]. Mice lacking IL-8 receptors, are less susceptible to form atherosclerosis and have fewer monocytes accumulated in vascular lesions [15].

Aspirin, an anti-thrombotic drug, is clinically used for the secondary prevention of transient ischemic attacks (TIA), ischemic stroke and coronary heart diseases, which are closely related to atherosclerosis [19,20]. However, to our knowledge, no effective drug has been found to induce regression of atherosclerosis. Weber et al. [21] showed that aspirin suppresses TNF-α-induced NF-kB activation in endothelial cells, to inhibit expressions of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, and monocyte adhesion to the endothelium.

This study was intended to examine aspirin's effects on the expressions of MCP-1 and IL-8 in TNF-α-stimulated HUVECs and to understand its possible mechanism of action in it's potential anti-atherosclerotic therapy.

2. Materials and methods

2.1. Materials

Aspirin was purchased from Sigma (St. Louis, MO), and U937 cells from Culture Collection and Research Center CCRC No. 68002 (Hsinchu, Taiwan). All reagents were of analytical grade.

2.2. Cell culture

We purchased HUVECs and endothelial cell growth medium (EGM-2, CC3156), from Clonetics (San Diego, CA), which contains 10% fetal bovine serum, hydrocortisone, hFGF-B, vEGF, R3-IGF-I, ascorbic acid, hEGF, GA-1000 and heparine. HUVEC which was used between passages 1 and 6, were maintained in EGM-2 medium in a humidified chamber containing 5% CO_2 at 37 °C. Cells were cultured in 6-well or 24-well plates until confluent, and were washed twice and incubated with serum-free medium for 12 h before different concentrations of aspirin (10, 100, 300, 500 μg/ml) were added. After being incubated for 12 h, cells were stimulated with TNF-α (10 ng/ml) (R&D Systems; Minneapolis, MN) for 24 h for MCP-1 and IL-8 expression. After incubation, the supernatants were collected for ELISA analysis, and the cells were used for RNA isolation.

2.3. RNA isolation and reverse transcription–polymerase chain reaction (RT–PCR) analysis

Total RNA was extracted from 1×10^6 cells according to manufacturer's instructions. Briefly, Trizol (1 ml) (Life Technologies, Grand Island, NY) was added to the plates to lyse the cells, and then the cells were transferred to the microfuge tube. Chloroform was added and total RNA was collected in the aqueous phase after centrifugation. Finally, RNA was precipitated by isopropyl alcohol, then washed and re-dissolved in DEPC treated water. The concentrations of RNA samples were measured with a spectrophotometer (GeneQuant II, Pharmacia Biotech) to determine the OD_{260} and $OD_{260/280}$ values. In total, 5 μg of RNA samples was reverse-transcribed with an oligo-dT primer to synthesize first-strand cDNA consecutively under 65 °C for 15 min, 25 °C for 10 min, 42 °C for 60 min, 95 °C for 10 min, using SuperScript reverse transcriptase (Life Technologies) and followed by storage of products at 4 °C.

cDNA was amplified by PCR with specific primers for MCP-1 and IL-8; primers for MCP-1 were 5′-CTGCCCTTGCTGTCCTCCTCTG-3′ (sense) and 5′-CTGCCGGCTTCGCTTGGTTA-3′ (anti-sense). Primers for IL-8 were 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′ (sense) and 5′-TCTCAGCCCTCTTCATCAAAAACTTCTC-3′ (anti-sense). The PCR conditions used for MCP-1 and IL-8 were based on previous reports [22,23]. For MCP-1, PCR consisted of 35 cycles. Each cycle consisted of 1 min for denaturation at 94 °C, 1 min for annealing at 59 °C, 2 min for elongation at 72 °C, and an additional 10 min for elongation at 72 °C after the 35 cycles. For IL-8, PCR consisted of 19 cycles, each consisting of a 45-s denaturation at 94 °C, a 45-s priming at 65 °C, and a 90-s elongation at 72 °C (except for a 10 min one during the last cycle). β-Actin was used as a internal control. PCR products were visualized by agarose gel electrophoresis. Photos were scanned by Scan Maker II sp and analyzed by Image-ProR Plus software.

2.4. MCP-1 and IL-8 analysis by enzyme-linked immunosorbent assay (ELISA)

Concentrations of MCP-1 and IL-8 were quantified using commercially available duoset ELISA development system (R&D Systems). ELISA plates were coated with specific mouse anti-human MCP-1 antibody (2 μg/ml) and mouse-anti-human IL-8 antibody (4 μg/ml). Diluted cell-free supernatants were added into the wells in duplicate, followed by adding secondary biotinylated goat anti-human MCP-1 antibody (100 ng/ml) and goat anti-human IL-8 antibody (20 ng/ml). After washing to remove unbound reagent, streptavidin-conjugated horseradish-peroxidase

was added and incubated for 20 min. After washing, a substrate solution (a 1:1 solution of H_2O_2 and tetramethylbenzidine) was added to the wells. Color development was stopped with 2N H_2SO_4, and the intensity of the color was measured at 540 nm on an ELISA plate reader (Emax, Molecular Device). The data were calculated according to a standard curve using a Softmax computer program. The sensitivity for MCP-1 and IL-8 were 6.25–1000 and 31.25–2000 pg/ml, respectively. Anti-human MCP-1 monoclonal antibody (R&D Systems) was added (50 μg/ml) to culture medium to neutralize the secreted MCP-1.

2.5. U937 adhesion assay

The adhesion assay was modified as described [21]; U937 cells were labeled with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acethoxymethyl ester (BCECF/AM, 10 μg/ml; Sigma, MO) for 30 min at 37 °C. RPMI-1640 with 2% FCS was added to stop the reaction, and cells were suspended in M199. HUVEC were cultured in 24-well plate until confluent, then different concentrations of aspirin were added and incubated for 12 h. After incubation, TNF-α (10 ng/ml) were added to the wells and incubated for another 24 h. Then HUVECs were co-incubated with 10^6 BCECF/AM-labeled U937 cells/well for 30 min at 37 °C. Non-adhering U937 cells were removed, and the 24-well plates were washed twice with M199. The plates were inverted and centrifuged for 2200 rpm 5 min to remove M199 medium. Cells were lysed in 0.1% Triton X-100 in 0.1 mol/l Tris. Fluorescence was measured with a F-4500 Fluorescence Spectrophotometer (HITACHI) (using excitation at 510 nm and emission at 531 ± 25 nm) and analyzed by F-4500 software. The area under emission wavelength calculated fluorescence intensity of adherent cells by F-4500 software. Anti-human MCP-1 and IL-8 antibodies were added (50 mg/ml) to culture medium to neutralize the secreted MCP-1 and to assess chemotaxis. Medium from untreated cells was used to determine basal migration.

2.6. Migration assay

The migration assay was modified as described [24]. HUVEC were seeded in the upper chamber of a transwell tissue culture insert (8 μm pore size) (Costar Cambridge, MA) until reach confluent density. Different concentrations of aspirin were added to the upper wells and incubate for 30 min at 37 °C, 5% CO_2 incubator. Medium from the upper chamber were aspirated and U937 cells (10^6 cells/100 μl/well) were added to the upper chambers. Recombinant IL-8 (50 ng/ml) and rMCP-1 (50 ng/ml) were added into the lower wells. Incubate the plates in a 37 °C, 5% CO_2 incubator for 1.5 h, M199 containing 0.5 mM EDTA were used for washing the U937 cells from the lower part of the membrane to lower wells. Cells in the lower wells were collected by centrifugation at 3000 × *g* for 5 min and direct cell counting under microscope.

2.7. Statistical analysis

We use Student's *t*-test to analyze the differences of continuous variables which were presented as the mean ± S.E.M. The differences were considered significant if *P* value was smaller than 0.05.

3. Results

3.1. Aspirin inhibited TNF-α-induced MCP-1 mRNA in HUVECs

By using specific MCP-1 primers, RT–PCR analysis detected a single band of the expected size, 297 base pairs (bp). As shown in Fig. 1, aspirin suppressed the induction of MCP-1 mRNA by TNF-α in HUVECs. The endogenous expression of MCP-1 mRNA was observed as shown in lane 1, Fig. 1, which is consistent with that of another study [25]. Cell morphology and MTT test (data not shown) showed aspirin did not affect the steady-state expression of β-actin in cells and also cell viability. The pH value did not change substantially for all doses of aspirin tested (data not shown). The negative controls for RT–PCR, either without cDNA or without reverse transcriptase were performed side by side.

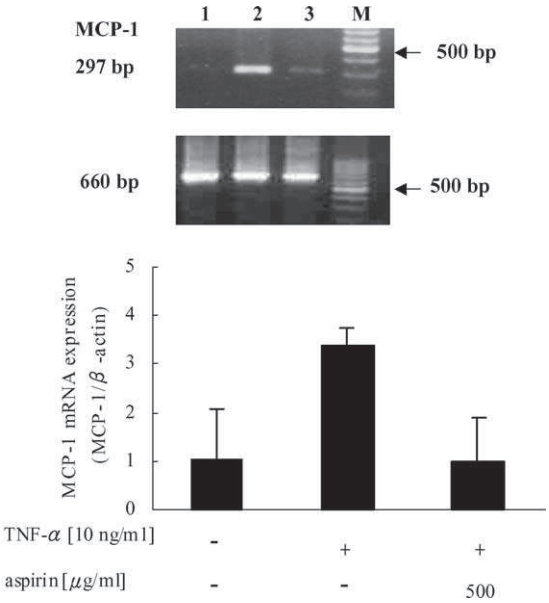

Fig. 1. Inhibition of MCP-1 mRNA expression by aspirin in HUVECs. HUVECs were incubated with serum-free medium for 12 h before different concentrations of aspirin were added, after 12 h incubation cells were stimulated with/without TNF-α (10 ng/ml) for another 24 h. Total RNA was extracted and analyzed by RT–PCR as described in Section 2. Lane 1, without TNF-α; lane 2, TNF-α (10 ng/ml) alone; lane 3, TNF-α + aspirin (500 μg/ml); M denotes molecular size marker. The lower panel is β-actin as internal control. Results are representative one of three independent experiments.

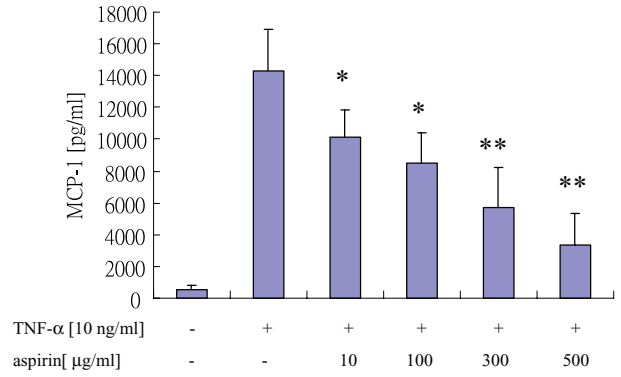

Fig. 2. Inhibition of MCP-1 secretion by aspirin in HUVECs. HUVECs were incubated with serum-free medium for 12 h before different concentrations of aspirin were added, after 12 h incubation cells were stimulated with/without TNF-α (10 ng/ml) for another 24 h. Culture supernatants were analyzed by ELISA as described in Section 2. Data are expressed as the mean ± S.E.M. of duplicate wells and are representative of five individual experiments. Significantly different vs. TNF-treated alone $^{*}P < 0.05$. Significantly different vs. TNF-treated alone $^{**}P < 0.001$.

3.2. Aspirin inhibited TNF-α-induced MCP-1 secretion in HUVECs

TNF-stimulated MCP-1 secretion was inhibited when cells were pretreated with aspirin in dose-dependent way (Fig. 2). Doses of aspirin as low as 10 μg/ml significantly ($P = 0.008$) reduced MCP-1 release (Fig. 2). Pre-treated cells with anti-MCP-1 antibody significantly ($P < 0.0001$) decreased the expression of MCP-1 (4664 ± 379.6 pg/ml, $n = 3$).

3.3. Aspirin inhibited TNF-α-induced IL-8 mRNA in HUVECs

By using specific IL-8 primers, RT–PCR analysis detected a single band of the expected size of 289 bp. As shown in Fig. 3, aspirin suppressed the induction of IL-8 mRNA in HUVECs treated with TNF-α. The β-actin gene was used as the internal control.

3.4. Aspirin inhibited TNF-α-induced IL-8 secretion in HUVECs

TNF-α-stimulated IL-8 secretion was inhibited (Fig. 4) when cells were pretreated with aspirin in dose-dependent way. Doses of aspirin as low as 10 μg/ml significantly ($P = 0.0146$) reduced IL-8 release. Pre-treated cells with anti-IL-8 antibody significantly ($P < 0.0001$) decreased the expression of IL-8 (9.84 ± 1.34 pg/ml, $n = 3$).

3.5. Inhibition of U937 cell adhesion to TNF-treated HUVECs by aspirin

The human premonocytic cell line U937 [26] has been extensively used to investigate leukocyte-endothelial cell

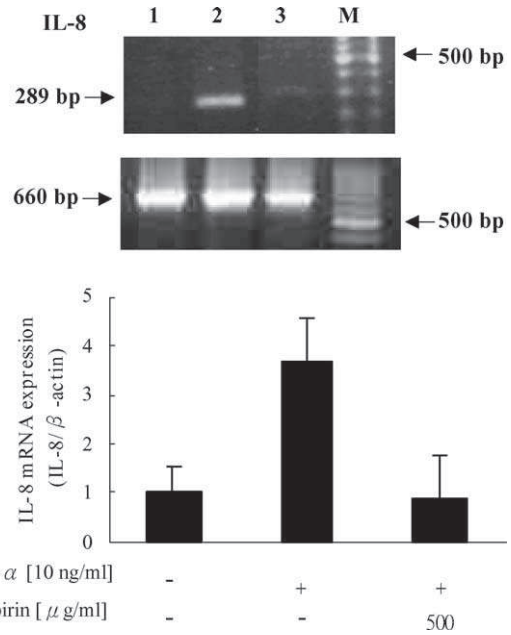

Fig. 3. Inhibition of IL-8 mRNA expression by aspirin in HUVECs. HUVECs were incubated with serum-free medium for 12 h before different concentrations of aspirin were added, after 12 h incubation cells were stimulated with/without TNF-α (10 ng/ml) for another 24 h. Total RNA was extracted and analyzed by RT–PCR as described in Section 2. Lane 1, without TNF-α; lane 2, TNF-α (10 ng/ml) alone; lane 3, TNF-α + aspirin (500 μg/ml); M denotes molecular size marker. The lower panel is β-actin as internal control. Results are representative one of three independent experiments.

interaction [27]. Studies demonstrated IL-8 and MCP-1 receptors, CXCR2 and CCR2B mRNAs were detected in U937 cells [28]. Adhesion of human premonocytic U937 cells to HUVECs, in our study showed TNF-α significantly

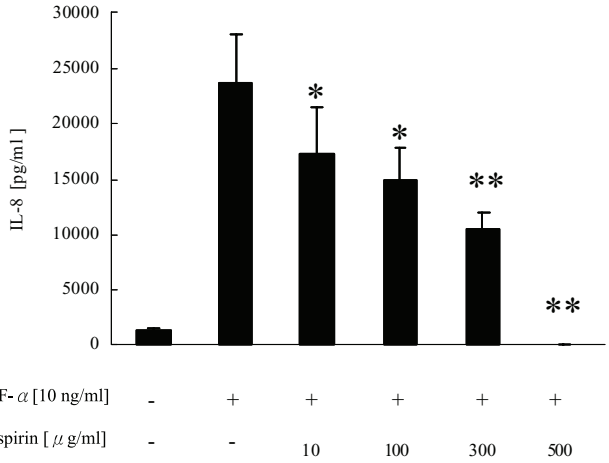

Fig. 4. Inhibition of IL-8 secretion by aspirin in HUVECs. HUVECs were incubated with serum-free medium for 12 h before different concentrations of aspirin were added, after 12 h incubation cells were stimulated with/without TNF-α (10 ng/ml) for another 24 h. Culture supernatants were analyzed by ELISA as described in Section 2. Data are expressed as the mean ± S.E.M. of duplicate wells and are representative of six individual experiments. Significantly different vs. TNF-treated alone $^{*}P < 0.05$. Significantly different vs. TNF-treated alone $^{**}P < 0.001$.

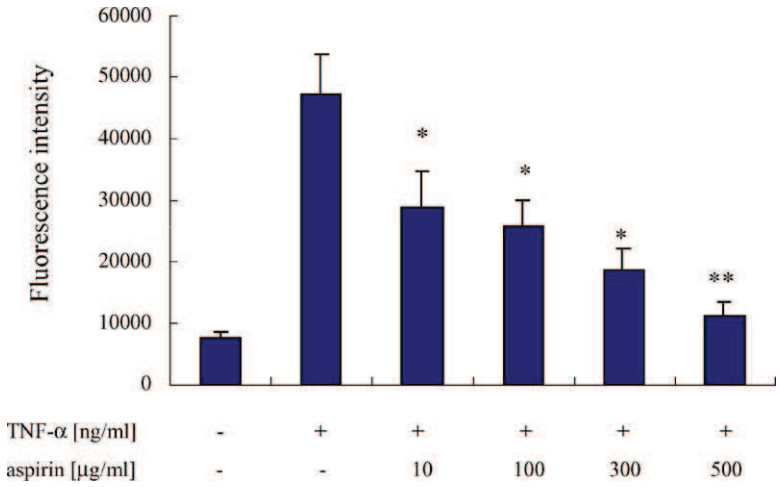

Fig. 5. Inhibition of monocyte (U937) adhesion by aspirin. HUVECs were incubated with serum-free medium for 12 h before different concentrations of aspirin were added, after 12 h incubation cells were stimulated with/without TNF-α (10 ng/ml) for another 24 h. Adhesion of fluorescence-labeled U937 cells was determined as described in Section 2. Data are expressed as the mean ± S.E.M. of five individual experiments. Significantly different vs. TNF-treated alone $^{*}P < 0.05$. Significantly different vs. TNF-treated alone $^{**}P < 0.001$.

($P < 0.001$) enhanced U937 adhesion. Aspirin induced a dose-dependent inhibition of TNF-induced adhesion (Fig. 5), which is consistent with its suppressive effect on MCP-1 and IL-8 induction. By adding of anti-IL-8 antibody for neutralization but not anti-MCP-1 antibody, the U937 cells show significantly less ($P = 0.017$) adhesion (31,371± 5548 FI) as compared to TNF-α stimulated alone (Fig. 5).

Our data suggest that aspirin may down-regulate TNF-stimulated IL-8 expressions and subsequent monocyte adhesion to endothelial cells.

3.6. Inhibition of U937 cell transendothelial migration by aspirin

At 300 and 500 μg/ml concentrations of aspirin significantly inhibit the U937 cell migration to the lower wells (Fig. 6). Pretreatment the HUVEC with anti-MCP-1 antibody (50 μg/ml) but not anti-IL-8 antibody will significantly decreased (2250 ± 675.2 cells, $P < 0.001$) the migration of U937 cells.

4. Discussion

Monocyte adhesion, migration, and accumulation from the vascular epithelium to the sub-endothelium play an important role in the pathogenesis of atherosclerosis [29]. MCP-1 and IL-8 expressions were found in lesions with monocyte infiltration [12,18]. Both increased MCP-1 and IL-8 levels have been observed in patients with acute coronary syndromes. The elevation of those and other possible chemokines can be used to predict overall atherosclerotic

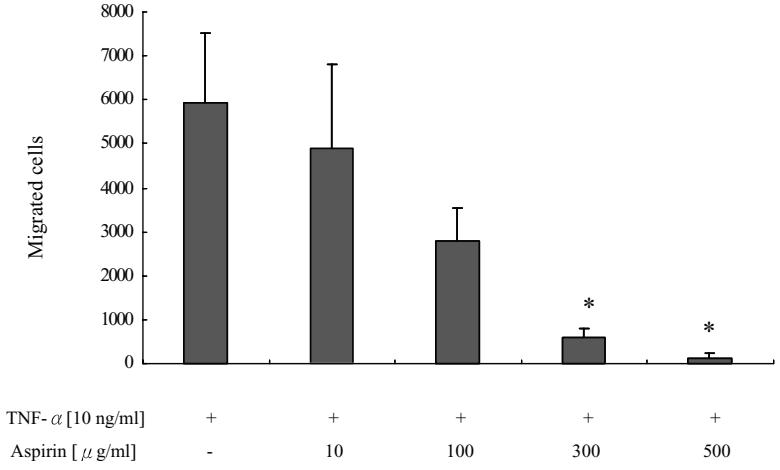

Fig. 6. Inhibition of U937 cell migration by aspirin. HUVECs were incubated at the upper wells of the transwell plates until confluency, different concentrations of aspirin were added, after 30 min incubation, cells were stimulated with/without TNF-α (10 ng/ml). U937 cells were added to the upper chamber wells and incubated for 1.5 h. The cells migrated to the lower wells were counted. Data are expressed as the mean ± S.E.M. of three individual experiments. Significantly different vs. TNF-treated alone $^{*}P < 0.05$.

burden [30]. As shown in Figs. 2 and 4 we showed that aspirin significantly inhibited TNF-α-induced expressions of MCP-1 and IL-8 in HUVECs and monocyte adhesion onto endothelial cells. According to previous studies [8,31], cytokine–activated endothelial cells may secrete monocyte-specific chemoattractant molecules, to amass monocytes at sites of vascular injury and/or inflammation such as atherosclerosis.

Gu et al. [32] found that the MCP-1-deficient mice deposit fewer lipids and have fewer macrophages within the aortic walls after LDL receptor-deficient mice have been fed with a high cholesterol diet. A study by Boring et al. [33,34] showed an overall decrease in atherosclerotic lesion size in mice lacking one of the MCP-1 receptors, CCR-2. Further, macrophages and monocytes were less significantly present in the aorta of CCR-2-deficient mice although the overall plasma cholesterol levels were unaffected by the CCR-2 genotype. These data from the literature suggest that a non-cholesterol-mediated effect on MCP-1 in developing atherosclerotic lesions. MCP-1 obviously plays a crucial role in initiating atherosclerosis by recruiting macrophages and monocytes to the vessel wall, and that the process of monocyte recruitment is a major determinant of lesion size.

U937 adhesion to endothelial cells is mediated by VCAM-1 and E-selectin expression [21]. Gerszten et al. [17] showed that MCP-1 and IL-8 rapidly convert initial rolling monocytes on transduced monolayers to adhere firmly by activating leukocyte integrins. They concluded that chemokines augment monocyte firm adhesion under flow conditions, and suggested that chemokines can start monocyte infiltration to the injured endothelium. Furthermore, data from Huo et al. [35] showed that except murine MCP-1, chemokine KC (mouse GRO-α, which appears to be the closest equivalent to IL-8), can trigger monocyte arrest on early atherosclerotic endothelium. The findings in our study, showed that only anti-human IL-8 antibody significantly blocked U937 adhesion to HUVEC and only anti-MCP-1 antibody significantly blocked U937 transendothelium migration. Based on these findings, we suggest that MCP-1 and IL-8 may be involved in U937 adhesion to HUVEC and migration. Taken together, we suggest that in addition to VCAM-1 and E-selectin, IL-8 may contributed to the U937 adhesion to the endothelials. Furthermore, MCP-1 may involved in U937 transmigration.

Chemokines in the CXC family, can be subdivided based on the presence or absence of the amino acid sequence Glu–Leu–Arg (ELR) preceding the first conserved cysteine amino acid residue. The ELR motif can interact ligand/receptor interactions on neutrophils and in regulate CXC chemokine-induced angiogenesis [33,34]. ELR^+ CXC chemokines such as IL-8, growth-regulated oncogenes GRO-α, GRO-β, GRO-γ, neutrophil activating protein-2 (NAP-2), epithelial cell-derived, neutrophil attractant-78 (ENA-78) and granulocyte chemotactic protein-2 (GCP-2), are potent angiogenic in vivo [36–38]. Contrariwise, ELR^- CXC chemokines, such as platelet factor-4 (PF-4), interferon-γ (IFN-γ)-inducible protein-10 (IP-10), monokine induced by IFN-γ (Mig) and stromal cell-derived factor-1 (SDF-1), are potent angiostatic factors [33,34]. Study showed the CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-mediated angiogenesis [39]. IL-8, shown to be an angiogenic factor in vivo and a chemotactic and mitogenic factor for vascular smooth muscle and endothelial cells in vitro, is markedly elevated in atherectomized specimens and may be a primary signal for angiogenesis in artherosclerotic tissue [38]. IL-8, which is found colocalized with vascular endothelial cells, supports a role for chemokines in atherogenesis, and, in turns, may contribute to developing and progressing of coronary atherosclerotic disease in humans [38].

Aspirin is widely used as an anti-inflammatory, antithrombotic, and even an anti-malignant agent, was found to inactivate the transcription factor, NF-kB, which contains a DNA-binding domain for consensus sequences of various cytokines and chemokines including MCP-1 and IL-8 [40–43]. The findings of our study showed that aspirin down-regulate MCP-1 and IL-8 not only at the mRNA but also at protein levels. These results are different from Amberger et al.'s work that aspirin inhibits MCP-1 mRNA expression by Northern blot analysis [43]. In examining aspirin's effects on TNF-α-induced expression of MCP-1 and IL-8 in HUVECs, we also found that aspirin suppressed both the mRNA and protein expression of MCP-1 and IL-8. In addition, aspirin is found to suppress monocyte adhesion and transmigration to endothelial cells. Taken all the findings together, we suggest that aspirin may inhibit atherosclerotic process by blocking the crucial step of monocyte adhesion and migration through suppressing MCP-1 and IL-8 expression in damaged or cytokine-stimulated endothelial cells.

Acknowledgements

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