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The effect of simvastatin treatment on TNF-alpha–induced inflammation in human vascular endothelial cells (HUVECs)

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Abstract: This study investigates the effects of simvastatin (SMV) on human umbilical vein endothelial cells (HUVECs) inflamed by tumor necrosis factor-alpha (TNF-a). Cells were treated with simvastatin (5 µM, 10 µM, and 50 µM) with/without TNF (20 ng/mL). The effects of simvastatin on the proliferation of cells were examined, and its cytotoxic effect was determined by lactate dehydrogenase (LDH) assays. Metalloproteinase expression (MMP-2 and MMP-9) levels of SMV at different concentrations were determined by qPCR, and protein levels were determined by Western blot. The increasing concentrations of SMV down-regulated the expression levels of MMPs. After stimulation with TNF-a, the effect of SMV at different concentrations on wound healing time in case of wound formation was examined. The results interestingly indicated that SMV delayed the closure time 2.5-fold when it was administered alone to HUVECs but did not change the wound closure time in inflamed conditions. The study suggests that inflammation is a required condition in wound healing, and in the absence of inflammation, SMV treatment delays wound healing in vitro.

Key words: Human umbilical vein endothelial cell, simvastatin, tumor necrosis factor-alpha, matrix metalloproteinase, wound healing
concentrations has not been investigated in detail after inflammation of HUVEC with TNF-α.

The wound healing model, also known as scratch assay, is commonly used in studies investigating the effects of cytokines and has become popular in recent years and is a relatively new technique that can be used to investigate wound healing and cell migration in vitro. The technique reproduces the wound by creating a gap in the confluent cell layer. Homeostasis, inflammation, migration-proliferation, and maturation occur during wound healing. Under the influence of cytokines, keratinocytes behind the wound edge begin to proliferate and migrate to the wound bed. This process includes cell migration, proliferation, and differentiation [14]. The possible effects of simvastatin on this cell migration in cells stimulated by TNF, a well-known proinflammatory molecule, have not yet been investigated.

This study aimed to investigate the possible effect(s) of SMV on TNF-α treated HUVECs and their underlying mechanisms. In the study, HUVECs were stimulated using TNF (20 ng/mL) and treated with different concentrations of SMV (5, 10, and 50 µM) to investigate the effect of MMP-2 and MMP-9 on mRNA and protein expression. Cell proliferation capacity was measured. Lactate dehydrogenase (LDH) activity was also monitored for toxicity assessment. In addition, a wound healing model was created. Using this model, the possible effects of different concentrations of simvastatin applied to TNF stimulated HUVECs on wound closure time were investigated.

2. Materials and methods
2.1. Materials
TNF-α was purchased from Biolegend (San Diego, ABD). Simvastatin was purchased from Santa Cruz Biotechnology (Teksas, ABD). They are solubilized in ultra ddH2O and dimethylsulfoxide (DMSO), respectively. Dulbecco’s modified eagle medium (DMEM), and penicillin/streptomycin were obtained from Capricorn Scientific (Darmstadt, Germany). TRIzol reagent was obtained from Invtrogen (Cat No: 15596-026, MA, USA).

NG dART RT kit was purchased from EURx (Cat No: E0801-02, Gdańsk, Polonia). BrightGreen 2X qPCR MasterMix-No Dye kit was from Applied Biological Materials Inc. Abm (Cat No: MasterMix-S, Richmond, Canada), cytotoxicity detection kit was purchased from Roche (Munich, Germany).

PVDF membranes were obtained from Merck Millipore (cat no. IPVH00010, CA, USA). MMP-2 and MMP-9 were purchased from St John’s Laboratories (dilution, PBS-T, 1:1000, cat. no. STJ94163 and STJ93257, respectively). Beta-actin was obtained from Elabscience (dilution, PBS-T, 1:3000, cat. no. E-AB-00156, Houston, USA). Goat anti-rabbit IgG H&L (HRP) was obtained from Abcam (ab97051; Abcam). PBS tablet (Merck Millipore, Darmstadt, Germany), Tween 20 was purchased from Sigma-Aldrich (Cat no: P1379, St. Louise, USA).

2.2. Cell line and cell culture
The study was conducted in accordance with the guidelines of the Institutional Ethics Committee. A commercially available human umbilical vein endothelial cell line (HUVEC) was used. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO2 in DMEM without FBS, containing 1% L-Glutamine (4.0 mM) and 1% penicillin-streptomycin, and glucose (4.5 g/L).

3 × 10^4/2000 μL cells were seeded into each well of 6-well sterile plates and cultured in an incubator for 24 h to adhere to the base and grow. Cells, whose medium was changed after washing with PBS twice, were monitored taking into account the scheduled times for the study (24 h for qPCR, 48 h for wound assay using Zencell System).

Cells were divided into groups: culture medium without any treatment as a control group; culture medium containing TNF-α (20 ng/mL) as positive control; culture medium containing only simvastatin (50 µM); culture medium containing TNF-α (20 ng/mL) with different doses of simvastatin (5, 10, and 50 µM) as the treatment group, respectively (Table 1).

These concentrations of simvastatin were chosen by reviewing articles as they were close to the concentration detected in the human body when administered therapeutically.

Following TNF-α stimulation, the effects of simvastatin treatment on cells were monitored at 12, 24, and 48 h. Total cell number, number of cells adhered to the plate, and number of cells detached from the plate were calculated as fold change compared to control group. Experiments were performed in duplicate for each condition.

2.3. Cytotoxicity analysis with LDH
Lactate dehydrogenase (LDH) activity was measured for toxicity assessment in cell culture. At the 24th hour after all treatments, 100 µL of the media in which the cells were contained were collected and Roche cytotoxicity detection kit was used to determine the LDH activity. The absorbance values obtained by the spectrophotometric method at 492 nm were recorded. Protein content was determined using a Pierce™ BCA Protein Assay kit (cat. no. 23225, Thermo Fisher Scientific, Inc.).

2.4. Polymerase chain reaction (PCR) analysis
0.5 mL of TRIzol reagent was added per well and the lysate was formed by mixing the cells at room temperature for 5 min. Lysates were taken into RNAase/DNAase-free Eppendorf tubes (1.5 mL). These tubes were centrifuged at 12,000 g, 4 °C for 10 min to collect supernatants and cellular RNA was isolated from the supernatant according to the kit instructions.
qPCR was used to analyze mRNA expressions (MMP-2, MMP-9). cDNA was also synthesized using the NG dART RT kit. The primer pairs (forward and reverse) used for MMP-2, MMP-9, and GAPDH are listed in Table 2.

The reactions were performed using a qPCR analyzer (Rotor-Gene Q, Qiagen, Germany) under the conditions of 10 min at 95 °C (1 cycle); 15 s at 95 °C; 60 s (40 cycles) at 60 °C. GAPDH values were used as an internal control to normalize expression data.

2.5. Western blotting
Proteins were extracted from HUVEC using Laemmle buffer containing proteinase inhibitor. The total protein amount was calculated using the Pierce™ BCA Protein Assay kit procedure. The samples (15 µg) were transferred to the PVDF membrane (0.45 µm) with a semidry transfer device (BIO-RAD) after electrophoresis in the prepared 10% polyacrylamide gel.

PBS-T containing 0.1% Tween 20 and 3% skimmed milk powder was applied for 1 h to reduce nonspecific binding to the membrane. The membranes were incubated on a shaker at 4 °C for 16 h by adding primary antibodies to MMP-2, MMP-9, and Beta-actin. After the incubation period was completed, the membranes were washed 3 times with 1X PBS-T solution for 5 min and incubated for 1 h with the secondary antibody Goat pAb to Rb IgG. Membranes were washed again with 1X PBS-T solution 3 times for 5 min. ECL solution was added to the membranes to see the protein bands and after 2 min in the dark, imaging was performed with the ChemiDoc ItR2 Imager (UVP) device. Bands were analyzed with the Image J program.

2.6. Wound healing assay
The HUVEC cell line was cultured at 10,000 cells/well/500 µL in a 24-well cell culture plate. After 4 days, a wound model was created by mechanical scratching using a 100-µL sterile tip. Next, the treatments shown in Table 1 were applied to examine the effects of TNF and simvastatin on HUVECs in wound healing model (Table 1). At this stage, a fresh medium (high glucose DMEM) containing 5% FBS was used. Cells were incubated for 48 h in the incubator and imaged using a 24-channel ZenCell Owl (Live Cell Imaging, InnoME GmbH, Bremen, Germany) device that allows real-time microscopic images of wound closure. The wound healing times (hours) were measured according to the migration of cells to fill the injured area is the first step in the wound healing process according to

Table 1. Treatment groups of the study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control*</td>
</tr>
<tr>
<td>2</td>
<td>TNF-a</td>
</tr>
<tr>
<td>3</td>
<td>SMV (50 µM)</td>
</tr>
<tr>
<td>4</td>
<td>TNF-a + SMV (5 µM)</td>
</tr>
<tr>
<td>5</td>
<td>TNF-a + SMV (10 µM)</td>
</tr>
<tr>
<td>6</td>
<td>TNF-a + SMV (50 µM)</td>
</tr>
</tbody>
</table>

*Control group includes only HUVEC
(Abbreviations: TNF-a, Tumor necrosis factor-alpha; SMV, simvastatin)

Table 2. Primer sequences.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2 human forward</td>
<td>TGACGGTAAGGACGGACTC</td>
</tr>
<tr>
<td>MMP-2 human reverse</td>
<td>ATACCTCACAGGGACCATTTG</td>
</tr>
<tr>
<td>MMP-9 human forward</td>
<td>CTGGGCAGATTTCCAAAACCT</td>
</tr>
<tr>
<td>MMP-9 human reverse</td>
<td>TACACCGCGATGAACTGAG</td>
</tr>
<tr>
<td>GAPDH human forward</td>
<td>TGATGACATCAAGAAGTTGGAAG</td>
</tr>
<tr>
<td>GAPDH human reverse</td>
<td>TCCCTGGAGGCCATGTGGGCCCAT</td>
</tr>
</tbody>
</table>

(Abbreviations: GADPH, glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metallo proteinase; G, guanine; C, cytosine; A, adenine; T, timine)
our measurement procedure, we recorded the hour the cells first interacted in the plot gap area.

2.7. Statistical analysis
Each experiment was repeated two times. Results were given as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM) and analyzed by ANOVA using Tukey correction for multiple comparisons. Statistical analysis was performed with the SPSS computer program v. 20.0, IBM corp., USA). p < 0.05 was considered statistically significant.

3. Results
3.1. Cell proliferation
Immunocytochemical analyses of HUVECs were performed. It was determined that these cells were induced by TNF, and then, as a result of combined treatment with SMV at different concentrations, the total number of cells, and cell numbers adhered to and detached from the plate surface were calculated (Figure 1). SMV treatment and TNF-a stimulation affected HUVEC proliferation in a dose- and time-dependent manner in 48-h cultures.

The total cell number increased continuously in all groups (except only SMV used) within 48 hours. However, high concentrations of SMV (50 µM) treatment in TNF-induced HUVECs significantly increased the cell number.

In the group using the highest concentration of SMV (TNF-a + SMV 50 µM), the number of cells adhering to the base of the plate did not change much over time. The number of these cells increased over time in groups using medium (TNF-a + SMV 10 µM) and low concentration

![Graph a](image1)

![Graph b](image2)
HUVECs proliferation has been stimulated with 20 ng/mL TNF-α and treatment with different concentrations of simvastatin for 12, 24, and 48 h. The graphics show the fold increase of the total cells (a), the cells adhered to the plaque surface (b), and the cells detached from the plaque surface (c). Experiments were carried out in duplicate for each condition. Bars represent the standard error of the mean. p < 0.05 was calculated for groups a, b, c vs control group (p = 0.048; 0.027; 0.003, respectively). *Statistically significant compared to the control (p < 0.05).

Figure 1. HUVECs proliferation has been stimulated with 20 ng/mL TNF-α and treatment with different concentrations of simvastatin for 12, 24, and 48 h. The graphics show the fold increase of the total cells (a), the cells adhered to the plaque surface (b), and the cells detached from the plaque surface (c). Experiments were carried out in duplicate for each condition. Bars represent the standard error of the mean. p < 0.05 was calculated for groups a, b, c vs control group (p = 0.048; 0.027; 0.003, respectively). *Statistically significant compared to the control (p < 0.05).

#Groups are control, TNF-α, SMV (50 µM), TNF-α + SMV (5 µM), TNF-α + SMV (10 µM), TNF-α + SMV (50 µM), respectively.

(TNF-α + SMV 5 µM). However, compared to the control group, SMV and TNF-α applied to HUVECs, especially TNF-α alone, decreased the number of cells adhering to the plate surface in all groups.

However, increasing the concentration of SMV treatment significantly increased the number of cells detached from the plate surface in TNF-induced HUVECs. In the group treated with high concentrations of simvastatin (TNF-α + SMV 50 µM), the number of cells detached from plate surface increased over the 24-h periods but not afterward.

3.2. Cytotoxic effects of simvastatin after inflammation of HUVECs with TNF-α
Toxicity assessment in cell culture was performed using an assay based on the measurement of lactate dehydrogenase (LDH) activity. The six groups (control and treatment groups) were compared to LDH activity in HUVECs (Figure 2). To determine the LDH activity in the media, the toxicity of the treatments was evaluated with the absorbance data obtained by the spectrophotometric method at 492 nm.

TNF-α inflammation of HUVECs significantly decreased cytotoxicity. While SMV treatment alone increased this cytotoxic effect, increased concentration of SMV treatment in TNF-α-stimulated HUVECs decreased the cytotoxic effect.

3.3. Effects of simvastatin on mRNA expression of MMP-2 and MMP-9 after inflammation of HUVECs with TNF-α
The mRNA expression levels of MMPs were evaluated. Changes in MMP expressions observed in treatment groups were compared with gene expression in the control group and calculated relative to the control group (Figure 3).

mRNA expression in HUVECs increased approximately 2-fold (MMP-9) and 20-fold (MMP-2) in TNF-induced cells after SMV treatment compared to the control group. While the effect of SMV on the release of the MMP-9 form in these stimulated cells was more modest, its effect on MMP-2 was found to be much greater.

When the effects of SMV concentrations (5, 10, and 50 µM) were examined, mRNA expression of MMP-2 and -9 genes were down-regulated gradually in a dose-dependent manner. According to the results obtained, it was determined that the 5-µM dose was the most effective dose for MMP-2 and -9. In addition, only SMV treatment quite up-regulated the mRNA expression of MMP-2 (p < 0.05).

3.4. Western blot
MMP-2 and -9 protein expressions were measured in HUVEC lysates by western blot analysis and the results are given in Figure 4.

Protein levels of MMP-2 and -9 were increased with TNF combined SMV treatment in HUVECs compared to the control group. While an increase was observed in the protein levels of MMP-2 with the increase of SMV concentration; MMP-9 protein levels first decreased and then a slight increase was observed. However, the greatest difference was experienced in the TNF stimulated HUVEC group, which received the highest dose of SMV.
**Figure 2.** The effect of SMV treatment on LDH activity present in culture medium in TNF-α stimulated HUVEC cultures. Data are presented as fold change of LDH levels compared to the controls. All experiments were performed in duplicate. Error bars represent SD. *Statistically significant compared to the control (p < 0.05).

**Figure 3.** Effect of SMV on MMP-2 and MMP-9 mRNA expression levels: a. MMP-2 and b. MMP-9 production by TNF induced cells after treatment for 48 h. The data was taken from two separate experiments, each performed in duplicate. Error bars represent SD. Statistically significant compared to the groups for MMP-2 (p = 0.033; p < 0.05). *Statistically significant compared to the control (p < 0.05).
3.5. Wound healing assay

The HUVEC cell line was cultured in a 24-well cell culture plate and 4 days later, using a 100-µL sterile tip, a wound healing model was generated and wound healing times were measured (hours).

The results obtained when different concentrations of simvastatin were monitored on wound healing of TNF-stimulated HUVEC cells are given in Figure 5. While a wound healing time of 35 h was found in cells treated with only SMV, simvastatin concentration did not make a difference in cells with inflammation with TNF.

Figure 4. Effect of SMV on MMP-2 and MMP-9 protein expression. a. MMP-2 and b. MMP-9 protein release after SMV treatment on TNF-a induced HUVECs. The data was taken from two separate experiments, each performed in duplicate. Error bars represent SD. Statistically significant compared to the groups for MMP-2 (p = 0.009; p < 0.05). *Statistically significant compared to the control (p < 0.05).
4. Discussion
This study aimed to investigate the effects of SMV at different concentrations on HUVECs induced with TNF. Our study includes cell proliferation, possible cytotoxic effects, possible effects on mRNA and protein expression and wound healing times were investigated. All these studies were performed in the case of SMV treatment, in the case of TNF stimulation, and in the conditions of SMV treatment at different concentrations after stimulation with TNF.

In the study, the effects of TNF-a and SMV on the proliferation of HUVECs and the attachment of cells to plates were investigated. It was observed that the total cell number decreased when only SMV was applied to HUVECs, but the cell number increased proportionally when SMV was applied to TNF-stimulated cells. The most significant decrease in the number of plate-bound cells was observed only when HUVECs were stimulated with TNF. The number of cells detached from the plate increased significantly with increasing SMV concentration in TNF-stimulated HUVECs. Increasing the concentration of SMV treatment decreased the surface-attachment properties of TNF-α-stimulated HUVECs. Studies showing that SMV induces p21- and p27-dependent G0/G1 cell cycle arrest in cell lines support our findings [7,15]. It has been shown that one of the reasons for the deterioration in this proliferation is SMV via vascular endothelial growth factor (VEGF) and it reduces dose-dependent proliferation [16]. In support of this, clinical studies have found a correlation between blood VEGF concentration and statin therapy [17].

LDH level was also discussed in our study as it is an indicator of cytotoxicity and cell death, and it was seen that the highest cell death was only with SMV application to HUVECs. Statins, of which SMW is an important member, have mechanisms of action such as inhibition of cell proliferation, cell cycle arrest, induction of apoptotic cell death, and inhibition of metastasis [18, 19]. The underlying mechanism of these effects is the inhibition of AMPK activation caused by statins, some protein synthesis, and suppression of cell proliferation [20,21]. It is also an expected result that the LDH level, that is, cytotoxicity, is low in cells stimulated with only TNF-α. In our study, we see that cell death is reduced again when we look at the effects of SMV application at different concentrations on HUVECs stimulated by TNF-a on the LDH level. In contrast to SMV, TNF-a is known to significantly

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**Figure 5.** The graph presents durations of closure time of the created clefts by HUVECs treated with/without TNF-a and different concentrations of SMV. For the migration characteristics of the cells, a slit pattern was established when the cells completely covered the well surface. The closure time of this created cleft was recorded using ZenCellOwl device for 48 h, real-time images of the cells were monitored with 1-h cycles with a ZenCellOwl microscope, and then the closing times were determined.

*Groups are 1. control, 2. TNF-a, 3. SMV (50 µM), 4. TNF-a + SMV (5 µM), 5. TNF-a + SMV (10 µM), 6. TNF-a + SMV (50 µM).*
Figure 6. Images of the wound healing of HUVECs. A wound scratch assay was performed using ZenCell. Images were given at the start of the wound formation (0 h) and wound healing time. Groups are 1. control, 2. TNF-α, 3. SMV (50 µM), 4. TNF-α + SMV (5 µM), 5. TNF-α + SMV (10 µM), 6. TNF-α + SMV (50 µM).
stimulate HUVEC proliferation at concentrations above 1 ng/mL [22]. On the contrary, a study also found that TNF-a can induce HUVEC apoptosis and inhibit HUVEC proliferation [23].

MMPs play an important role in physiological and pathological states involving inflammation, and inflammatory-related functions, including the activity of cytokines [24]. Therefore, in the study’s next step, the effect of SMV treatment after TNF stimulation on MMP-2 and MMP-9 expression and protein expression in HUVECs was examined. Study results showed that SMV treatment administered to HUVECs after TNF stimulation down-regulated genes in both MMP-2 and 9 in HUVECs in a dose-dependent manner as the concentration increased. This decrease may be responsible for increased number of cells detached from the plate surface. However, although we observed a decrease in MMP-9 levels, the differences between the groups were not statistically significant. Cytokines, including TNF, are known to be induced by MMPs and induce inflammation [25]. Expression of MMPs in different cell types has also been shown to be induced by an extracellular stimulus such as TNF. The mechanism underlying the regulation of TNF and MMP expression is related to c-Src, MAPK, and NF-kB in metabolic pathways [26]. Statins, on the other hand, have antiinflammatory effects and down-regulate the expression of MMPs. Lipophilic statins such as simvastatin more than hydrophilic statins such as pravastatin were found to down-regulate MMP-9 activity, which is consistent with our study results. Another study, including some of the authors, found that SMV also down-regulated the MMP-2 and MMP-9 genes in the interleukin 1β-induced SW1353 chondrosarcoma cell line [27]. In another study in which they looked at the possible effects of 1.25(OH)2D3 in human chondrocyte and SW1353 cells stimulated with TNF-a, MMP-1, -2, -3, -9, and -13 mRNA expressions were again decreased with 1.25(OH)2D3 treatment [28]. In our study, we can say that increasing the concentration of SMV treatment reversed the effects of MMP-2 and MMP-9 on mRNA expression levels in TNF stimulated HUVECs. These results are very significant to offer new strategies for intervention with inflammatory diseases without blocking the beneficial effects of MMPs.

Bernds’ study found that SMV increased the proteolytic capacity of MMP-2 and MMP-9 [29]. Our study observed a similar situation in the MMP-2 level with SMV administration in cells stimulated with TNF-a. A small decrease in MMP-9 followed by an increase in protein expression was observed in the same way. Because of the increased expression and activity of these MMPs in tumors, each factor affecting this expression is critical. Therefore, the results of this study show that SMV treatment can suppress tumor progression and invasion by down-regulating specifically MMP-9 levels in TNF-stimulated HUVECs. These findings may also contribute to developing new preventive treatments for different diseases.

Wound healing researches are useful tools to develop therapies to heal wounds in a shorter period, avoiding negative consequences such as infection and scarring. In our study, we conducted a time study on wound healing by creating a wound model to determine the dose-dependent potency of SMV on cell motility. According to the results of our research, wound closure time increased 2.5 times in cells treated with only SMV. However, it was observed that SMV concentration did not affect the closure time in HUVECs stimulated with TNF-a.

Wound healing is a dynamic and complex process involving coordinated events such as proliferation, hemostasis, inflammation, and revascularization [30]. Statins have been recognized as a new therapeutic modality for wound healing due to their various pleiotropic potency, independent of their lipid-lowering effects [31]. It has been suggested that these broad-spectrum pleiotropic effects of statins are related to their antiinflammatory, antioxidant, and immunomodulatory properties and ability to improve reperfusion [32-35]. The possible effects of statins on wound healing have not yet been adequately studied. The prognostic effects of statins in the wound healing process are rational and the results are promising. However, our study observed that high statin administration doses created a negative result interestingly and prolonged wound closure time. Interestingly, however, administration of simvastatin after inflammation reduced the wound healing time of simvastatin. Similar to our study, some studies found that SMV delays wound healing by inhibiting VEGF protein secretion and disrupting proliferation. SMV, along with many statin types showing significant antiproliferative and antimigratory properties, inhibits isoprenylation and cell growth factor (HGF)-dependent cell migration [7]. On the other hand, several other studies have shown that statins aid wound healing by increasing endothelial formation by inducing NO synthase, increasing VEGF release, and reducing oxidative stress [36-39]. Considering the different pleiotropic and prognostic effects of SMV in the wound healing process, it should be taken into account that the possible effect of SMV on wounds in different tissue types may also differ.

5. Conclusion
This study suggests that SMV treatment, with/without TNF stimulation, affects cell proliferation, MMP mRNA and protein expression and wound healing in HUVECs differentially. That research should be applied by simulating various pathological processes to fully comprehend statins’ acting mechanisms.

Conflict of interest
The authors declare no conflicts of interest.
References


