THE EFFECT OF CILOSTAZOL ON THE TISSUE FACTOR EXPRESSION IN HUVECs AND HUMAN MONOCYTES

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감사의 글

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바쁘신 와중에도 자상하게 조언을 해주시고 교정을 해주신 김수기 교수님께도 진심으로 감사 드립니다. 실험이 진행되는 과정에서 격려를 아끼지 않으셨던 이승환, 유병수 교수님, 그리고 최경훈 교수님께도 감사 드립니다.

실험을 직접 진행 하며 기본적인 지식을 알려주신 김근하 선생님께도 감사 드립니다.

마지막으로 항상 저에게 사랑과 행복을 주시는 부모님과 기쁨을 나누고 싶습니다.

2004 년 7 월

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ABSTRACT

The Effect of Cilostazol on the Tissue Factor Expression in HUVECs and Human Monocytes

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Tissue factor (TF) is a potent initiator of the coagulation cascade situated within the vessel wall as well as an important key sentinel in the pathogenesis of thrombosis and restenosis after balloon angioplasty. While TF is expressed not only on endothelial cell but also on innate immunocytes such as monocytes and macrophages, pathogenic environment culminates TF expression and release, leading to acute coronary syndrome or restenosis after balloon angioplasty. Recent basic studies focus on the therapeutic inhibition of tissue factor.

Cilostazol, even though it is classically known as AMP-PDE inhibitor, is clinically effective because of its possible antiplatelet, vasodilating,

antimitogenic, and cardiotonic action. However, there were no known documents regarding usage or significance of cilostazol in direct relation with TF expression. To clarify this issue, we primarily examined the effect of the cilostazol on TF expression in TNF-α stimulated HUVECs and LPS-stimulated monocytes._HUVECs and CD14+ monocytes isolated from whole blood of healthy volunteers were incubated for 30minutes with or without cilostazol (1, 10, 100 μM). Thereafter, HUVECs were treated with TNF-α (10 ng/ml) and CD14 + monocytes with LPS (1 μg/ml), and incubatedα for 4 hours for RT-PCR or for 24 hours for Western blotting. As a result, expression of TF-mRNA and TF in TNF-α stimulated HUVECs was down regulated by cilostazol pretreatment in dose dependant manner. This down regulation in TF-mRNA and TF protein was similar in CD14+ monocytes.

In conclusion, these data indicate that cilostazol act as a TF inhibitor on HUVECs and monocytes by down regulating the TF-mRNA and TF protein expression.

Key words: cilostazol, tissue factor, monocyte, HUVECs

The Effect of Cilostazol on the Tissue Factor Expression in HUVECs and Human Monocytes

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I. Introduction

Tissue factor (TF) is a recognized critical determinant of thrombotic responses in vivo. It is a transmembrane glycoprotein that initiates blood coagulation by binding factor VII with high affinity, thereby promoting factor X activation, thrombin generation, and fibrin formation (1, 2). Under normal circumstances, endothelial cells express no or minimal amounts of TF. However, TF expression is significantly increased with endothelial and monocytes activation

(3, 4). Many evidences have suggested that the accumulation of TF in atherosclerotic plaque plays a major role in determining plaque thrombogenicity (5, 6). In that context, it is rational that TF plays a key role in triggering thrombin generation after angioplasty relating to acute thrombotic complication or long-term restenosis.

Cilostazol (Otsuka Pharmaceutical Co, Ltd) is a new antiplatelet agent that increases the concentration of cAMP within platelets by selectively blocking type III phosphodiesterase, thereby inhibiting platelet aggregation (7), and causing vasodilation (8). Furthermore, several animal studies have shown that this drug also inhibits intimal proliferation in injured arteries by inhibiting smooth muscle cell proliferation (9). The beneficial effects of cilostazol on the apoptosis in the HUVECs damaged by LPS and restenosis in human were reported recently (10, 11).

Taken together, cilostazol may play a role of vasculopathy inhibitor. However, there was no data about the effect of cilostazol on the TF, which is an important initiator of thrombin generation causing acute thrombotic occlusion and long term restenosis, in human cells.

Under the hypothesis that cilostazol may exert an anti-TF action, we attempted to demonstrate the effect of cilostazol on the expression of TF-mRNA and TF protein in TNF-α stimulated HUVECs and LPS-stimulated monocytes.

II. Methods

1. Isolation of HUVECs

Human endothelial cells were isolated from human umbilical cord veins (HUVEC) by collagenase digestion method and grown to confluence in 0.2% gelatin coated 75 cm^2 (Nunc) flasks at 37 ℃ in an atmosphere of 95% air and 5% CO2 in endotoxin-free complete medium. HUVEC were characterized by

their typical morphology at confluence and positive immunofluorescence stain by using a monoclonal antibody specific for von Willebrand factor (figure 1).

2. Isolation of monocytes

Human monocytes from fresh peripheral blood were obtained from normal healthy volunteers. Mononuclear cells were isolated by centrifugation through histo-hypaque 1077 (Sigma, St Louis, MO, USA) and negatively selected with anti-CD 14 immunomagnetic beads using a MACS cell separation system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), obtaining a 97.87% monocyte preparation (figure2). Flow cytometry was performed by CD14-FITC conjugated Antibody (BD, Franklin Lakes, NJ USA).

3. Detection of TF-mRNA by RT-PCR

HUVECs and CD14+ monocytes cultured in 24 well plates, prepared as described above, were incubated for 30 minutes with or without cilostazol (1,

10, 100 μM). And then, HUVECs were treated with TNF-α (10 ng/ml) and CD14 + monocytes with LPS (1 μg/ml), and incubated for 4 hours for RT-PCR.

After 4hours incubation, total RNA was prepared by Trizol reagent (Invitrogen, Carlsbad, California, USA), and 1g of total RNA was reverse transcribed in MoMLV (Promega, Madison, WI, USA). TF and GAPDH were examined by PCR amplification to prove the cells in lanes to be same quantity.

4. Cell culture and protein extraction for Western blot analysis

Confluent endothelial cell monolayer and monocytes cultured in 6 well plates were treated without or with varying concentration of cilostazol (1, 10, 100 μM) for 30min. Then, HUVECs were treated with TNF-α (10 ng/ml) and monocytes were treated with LPS (1 μg/ml) to induce TF expression. After 24 hours incubation, the cells were washed twice with cold phospho-buffered saline (PBS). Cells harvested from wells by gentle scraping with a rubber policeman (Fisher Scientific, Pittsburgh, PA) were immediately sedimented.

Cell pellets were lysed at room temperature with RIPA buffer (Tris-HCl pH 8.0, 1% Triton-X, 150 mM NaCl, 0.5% deoxycholate, 1 mM PMSF). Protein concentration was determined according to Bradford utilizing Coomassie brilliant blue G-250 (Bio-Rad Labs, Richmond, CA).

5. Western blot analysis

Cell lysate (40 μg of protein) were boiled for 5 min in sample buffer, loaded, and electrophoresed in 12% Tris/glycine SDS-PAGE gel (Hoefer Scientific Instructions) using a Trans-blot SD transfer cell (Bio-Rad). Blots blocked 1hr at room temperature with a 1% blocking solution (1% powdered milk in 0.1% Tween-PBS solution) were incubated with anti-HUMAN TF antibody (American Diagnostica, CT, USA) for overnight. Blots were washed while shaking gently and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated anti-mouse IgG (Serotec, Kidlington, Oxford, UK). Membranes were then washed and recorded utilizing the enhanced chemiluminescence's

detection system (ECL, Amersham, UK) followed by exposure onto Kodak film. Blots were quantitated by VersaDoc imaging system (Bio-Rad). To prove the cells in lanes are same quantity, SDS-PAGE for TF and Tubulin was performed simultaneously.

III. Results

1. TF-mRNA expression in HUVECs and monocytes

TF-mRNA expression in HUVECs was suppressed by 6% with 1 μM, 78% with 10 μM, and 97% with 100 μM of cilostazol compared to the control expression of TF-mRNA without cilostazol in the pretreatment of TNF-α (10 ng/ml). As shown in figure 3, we can see that TF expression is suppressed in a dose dependent manner. Also in monocytes, the degree of suppression with cilostazol pretreatments was 13%, 79%, and 98%, respectively in proportion to the concentration of cilostazol on TF PCR as manner as HUVECs (figure 4).

2. Western blot analysis of tissue factor in HUVECs and monocytes

As shown in figure 5, TF expression in HUVECs is suppressed by 28% with 1 μM, 79% with 10 μM, and 97% with 100 μM of cilostazol compared to the control expression of TF without cilostazol in the pretreatment of LPS (1 μg/ml).

Also in monocytes, the degree of suppression with cilostazol was 13%, 79%, and 98%, respectively, in proportion to the concentration of cilostazol as manner as HUVECs (figure 6).

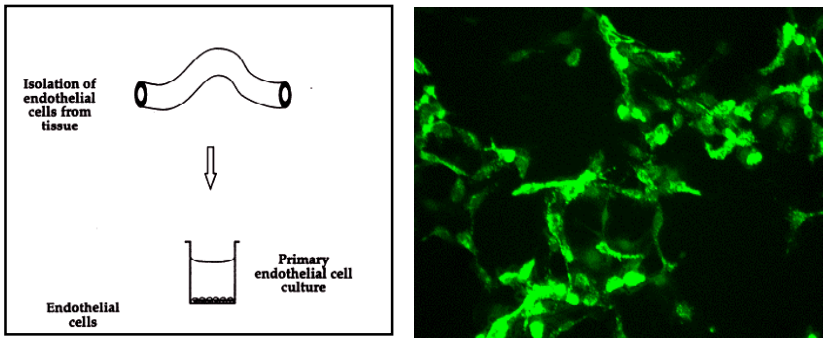


Figure 1. HUVECs stained with FITC-conjugated anti-vWF monoclonal antibody. Endothelial cells were isolated from human umbilical cord veins (HUVEC) by collagenase digestion method. HUVEC were characterized by their typical morphology at confluence and positive immunofluorescence stain by using a monoclonal antibody specific for von Willebrand factor.

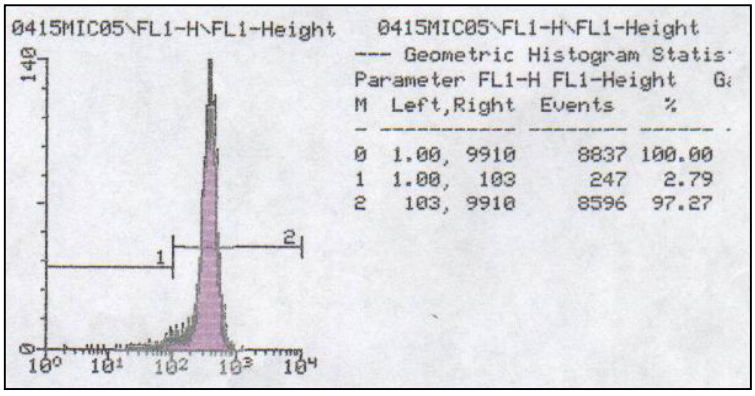
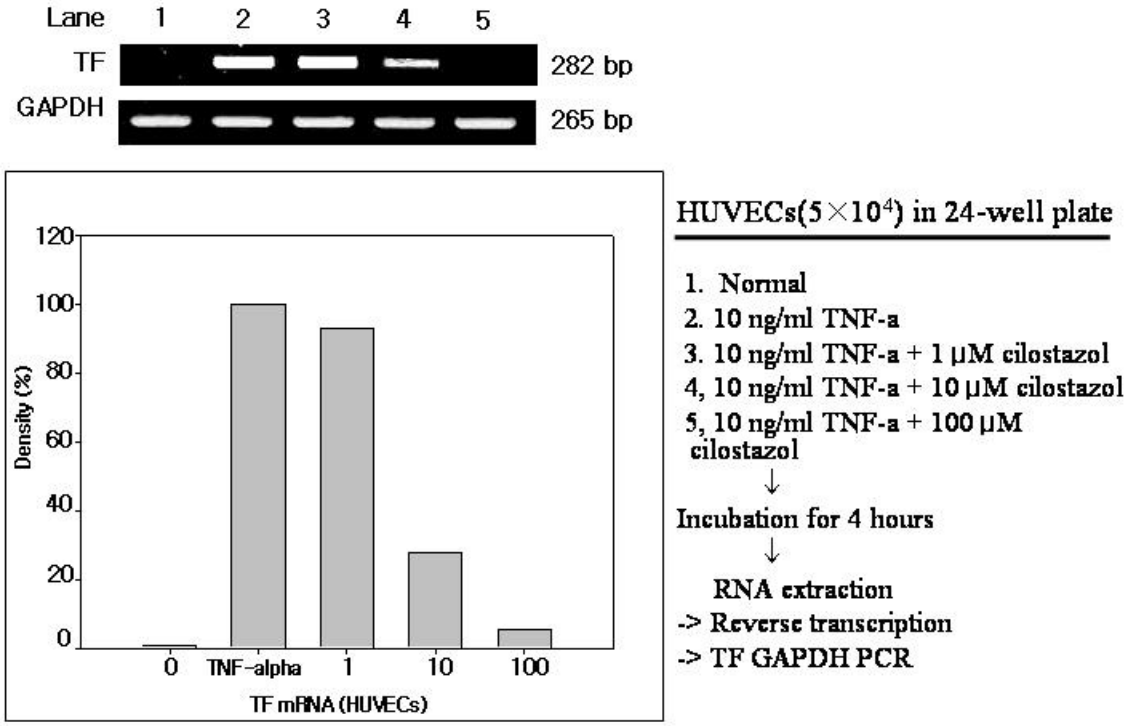


Figure 2. Monocytes are fluorescently stained with CD14-FITC. Mononuclear cells were isolated by centrifugation through histo-hypaque 1077 (Sigma, St Louis, MO) and negatively selected with anti-CD 14 immunomagnetic beads using a MACS cell separation system (Miltenyi Biotec GmbH), obtaining a 97.9% monocyte preparation. Flow Cytometry was performed by CD14-FITC conjugated Antibody.


Lane 1 2 3 4 5
TF
GAPDH
282 bp
265 bp
120
100
80
60
40
20
0
Density (%)
0 TNF-alpha 1 10 100
TF mRNA (HUVECs)
HUVECs(5×10⁴) in 24-well plate
1. Normal
2. 10 ng/ml TNF-a
3. 10 ng/ml TNF-a + 1 μM cilostazol
4, 10 ng/ml TNF-a + 10 μM cilostazol
5, 10 ng/ml TNF-a + 100 μM cilostazol
↓
Incubation for 4 hours
↓
RNA extraction
-> Reverse transcription
-> TF GAPDH PCR

Fi

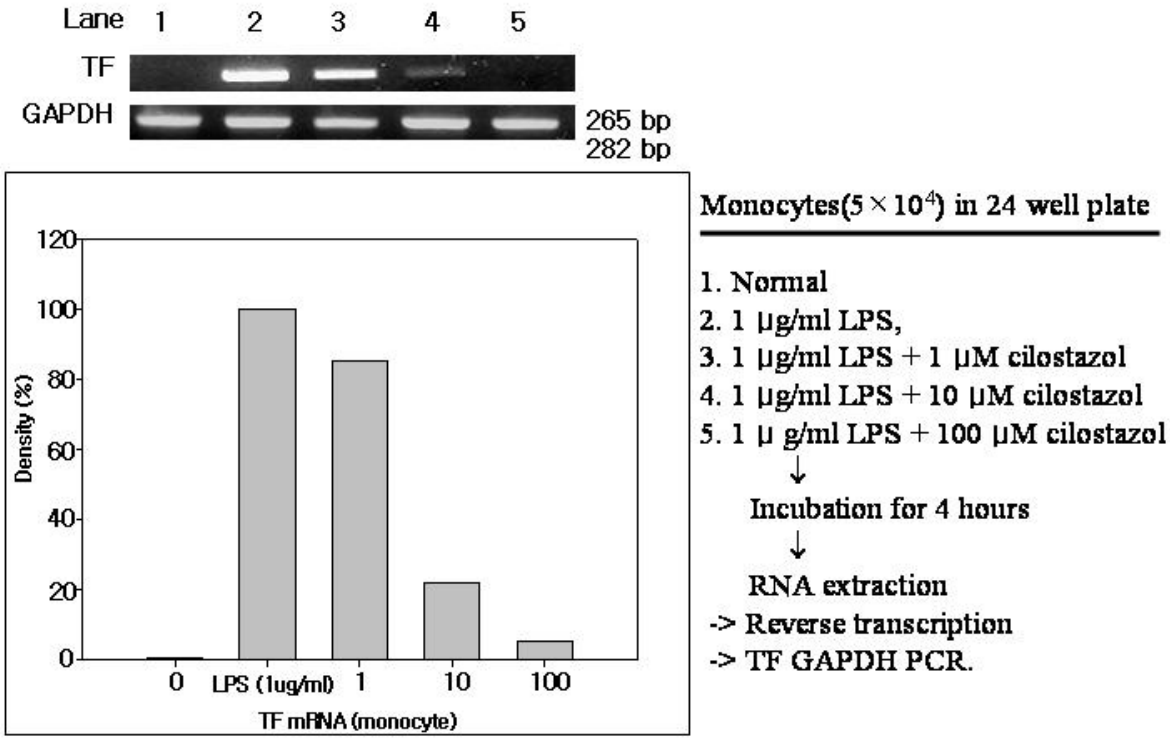


Figure 4. Inhibitory effect of cilostazol on LPS-induced TF-mRNA expression in monocytes

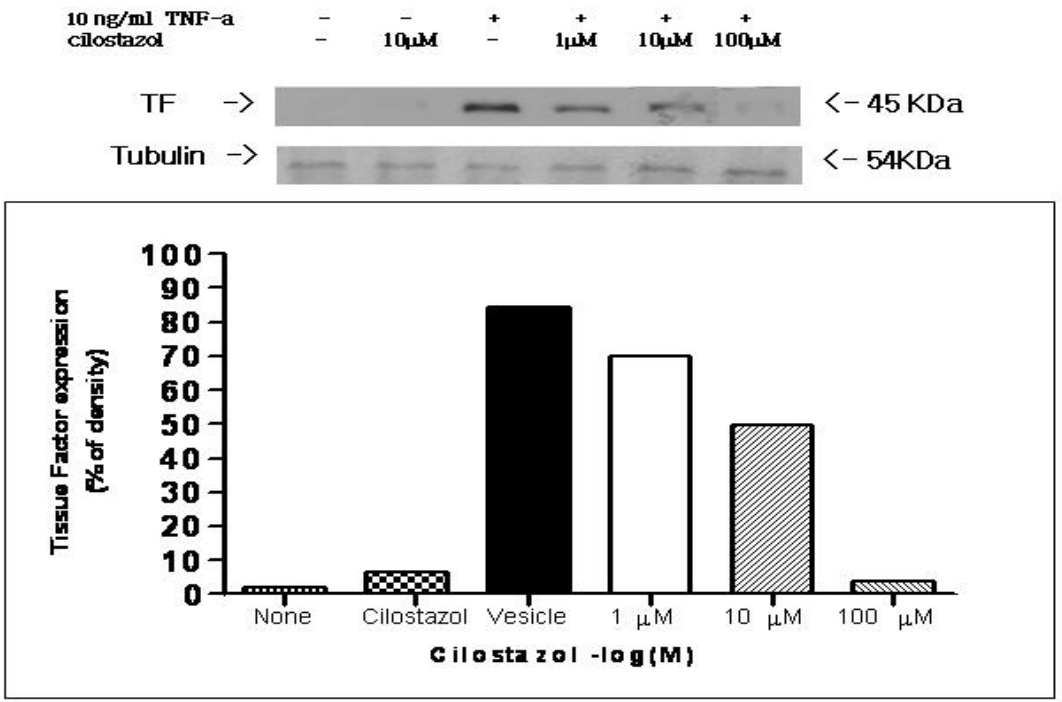


Figure 5. Western blot analysis of tissue factor in HUVECs

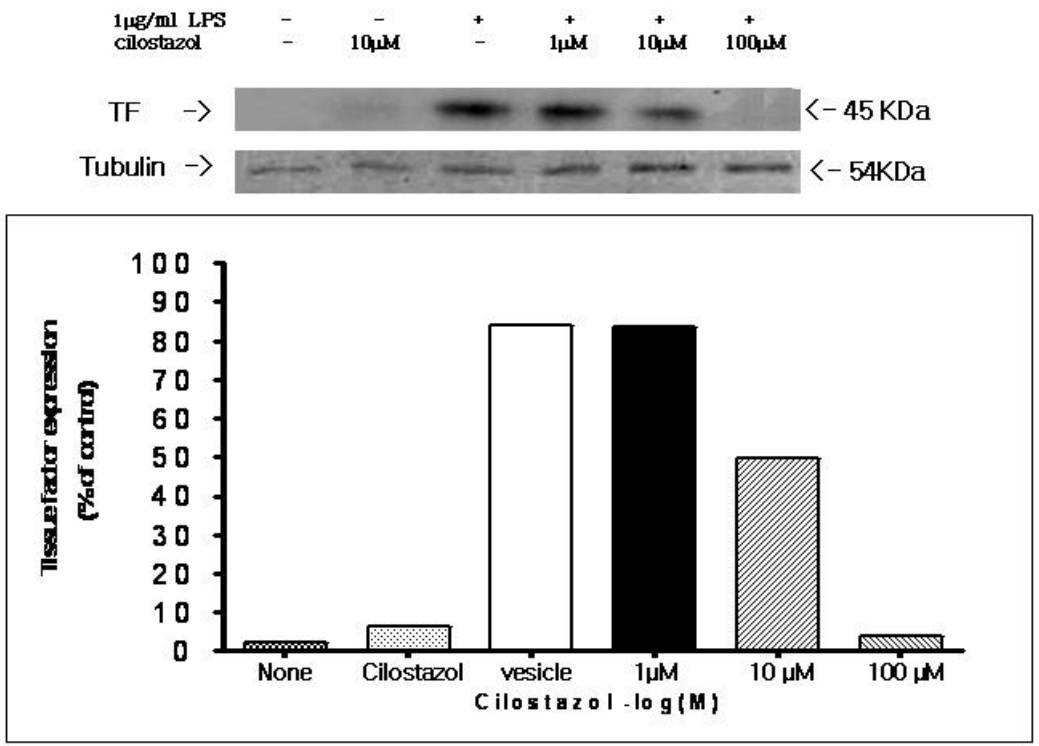


Figure 6. Western blot analysis of tissue factor in monocytes

IV. Discussion

Cilostazol, even though it is classically known as AMP-PDE inhibitor, is clinically effective because of its possible antiplatelet, vasodilating, and antimitogenic action. However, there were no known documents regarding usage or significance of cilostazol in direct relation with TF expression. To clarify this issue, we primarily examined the effect of the cilostazol on TF expression in HUVECs and monocytes.

This study demonstrates for the fist time that cilostazol reduces the TF-mRNA and protein expression of endothelial cells and monocytes stimulated by TNF-α or LPS in a dose dependant manner.

Despite clear down-regulation of cilostazol on monocytes and HUVEC as shown in Fig 3-6, TF suppressive mechanism remains to be elucidated. As plausible explanation for TF suppression, it might be intimately linked with intracellular cyclic AMP concentration. Supporting this, it was reported that

lysophospholipid. LysoPC (Lysophosphatidylcholine**)** inhibited the TF activity of Mono Mac-6 cells to a similar extent as in the monocytes. Induction of TF-mRNA expression by LPS tended to be partially reduced by the lysophospholipid. Preincubation with lysoPC increased monocytic cAMP levels. Inhibition of adenylyl cyclase by pretreatment with 29-deoxy-39-adenosine monophosphate partially reversed the inhibition of TF activity promoted by lysoPC. LysoPC markedly decreases LPS-mediated TF expression of human monocytes, the effect probably being mediated by both transcriptional and posttranscriptional mechanisms (12).

Additional report showed that prostacyclin analogues inhibited TF expression in the human monocytic cell line THP-1 via a cyclic AMP-dependent mechanism (13). Despite structural dissimilarity between cilostazol and other compounds, these finding suggest similarity or analogy with our finding, indicating that cilostazol act as TF inhibition in transcriptional and posttranscriptional level. Synthesizing these finding, it may be hypothetically

generalized that TF expression may be dependent upon the intracellular concentration of cAMP. In this point, it may be hypothesized that cilostazol will lower intracellular cyclic AMP via PDE action, leading to TF down-regulation in pre and posttranscriptional level. To confirm this hypothesis, further experiment will be warranted. However, still the molecular signaling mechanism implicated with TF inhibition of cilostazol is in mystery. Some report hinted that prostacyclin derivatives similar to TF inhibition of cilostazol prevented the fibrotic response to TGF-β by inhibiting the Ras/MEK/ERK pathway. (13)

But, this report had a clear limitation in that point of difference of target cell. Thus, it will be interesting to elucidate the intracellular signaling cascade implicated with TF inhibition triggered by cilostazol or analogous compounds.

As the well-known suppressors of TF expression, there are dilazep dihydrochloride, dipyridamole, pentoxifylline, several angiotensin-converting

enzyme inhibitors, or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (14-21). These TF inhibitors own their unique, differential action mechanism. For instance, dilazep, which inhibits antibody-induced monocyte TF activity in a dose-dependent fashion, but has no effect on TF-mRNA expression (14, 15). It means that dilazep inhibits the increased expression of monocyte TF activity at a posttranscriptional level (16). In contrast, several angiotensin-converting enzyme inhibitors (captopril, imidapril, and fosinopril) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (simvastatin, fluvastatin) significantly inhibit LPS-induced monocyte TF activity, antigen expression, and gene transcription similar to the cilostazol (17-21). Given this, our data clearly suggest that cilostazol may own different mechanism of TF inhibition when compared with other TF suppressors.

The in vitro effective arrays of cilostazol concentration used in our study were relatively higher than the clinically achievable in vivo concentration. In

general, however, the blood concentration achieved by 200 mg of oral cilostazol daily corresponds to 3 to 5 μM, thus the cilostazol effect was examined in the acceptable range (22). In our study, TF-mRNA expression in HUVECs was suppressed by 6% with 1 μM, 78% with 10 μM, and 97% with 100 μM of cilostazol. Also in monocytes, the degree of suppression with cilostazol pretreatments was 13%, 79%, and 98%, respectively. In Western blot analysis, TF expression in HUVECs was suppressed by 28% with 1 μM, 79% with 10 μM, and 97% with 100 μM of cilostazol and in monocytes, all of a degree (17, 49, and 98% suppressions, respectively). Therefore, it may be rational that at low concentration cilostazol rarely inhibit the expressions of the TF and TF-mRNA. However, the local tissue levels of the compound might be higher than the free concentration in plasma because of the lipophilicity of the drug. In that context, our finding has a clinically important implication. That is, elucidating the role of cilostazol as potential TF inhibitor confers insight into new development of TF inhibitor at clinically achievable concentration.

V. Conclusion

In conclusion, this study shows that cilostazol is effective in reducing the TF expression of endothelial cells or monocytes stimulated by TNF-α or LPS in a dose dependant manner. These data indicate that cilostazol has a potential to act as a TF inhibitor on HUVECs and monocytes, and that cilostazol might be effective in reducing the acute thrombotic complication of PTCA and inhibiting restenosis after PTCA.

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국문요약

HUVECs와 Monocytes에서 TF 발현에 cilostazol이 미치는 영향

<지도 윤 정 한 교수>

연세대학교 대학원 의학과

정 현 숙

조직인자 (Tissue factor: TF)는 혈관벽 내에 존재하여 혈액응고 과정을 개시하는데 매우 중요하며, 혈관성형후의 혈전생성과 재협착에도 관여하는 것으로 알려져 있다. TF는 혈관내피세포뿐만 아니라 단핵구, 대식세포와 같은 고유면역세포에 발현하며, 특히 염증과 같은 병적인 환경에서는 TF발현이 현저히 증가하며 이로 인해 급성 관상동맥질환이나 혈관성형후의 재협착 등의 직접 원인이 된다. 그러므로 최근에 TF를 표적으로 이 인자의 발현을 제어하는 약물요법 등이 많은 관심을 끌며 개발되고 있다.

본 연구에서 사용한 cilostazol은 AMP-PDE 억제인자로서 항 혈전, 혈관확장, 심근수축등 순환기계 작용 등이 잘 알려져 있다. 임상적으로 널리 사용되나 순환기계 미치는 영향 중 특히 TF 발현과 관련 지어 cilostazol의 시험관 및 생체 실험 및 그 중요성에 대해서 논의된 보고는 전무하다. 이에 이의 연관성을 입증하는 기초연구로

HUVECS 와 monocyte에서의 TF 발현에 관한 cilostazol의 효과를 검증하고자 본 연구를 시도하였다.

인체 제대혈관내피세포(HUVECS: human umbilical endothelial cell)과 단핵구 (Monocyte)을 건강한 성인으로부터 분리, 초벌배양(primary culture)을 한 후에 첫번째로 TF의 mRNA 발현을 보기위해서 PCR을 시행하였고 둘째로 TF 단백 발현을 규명하기 위해서 SDS-PAGE 법을 시행한 후에 TF, tubulin Ab를 이용하여 웨스턴 블롯법(Western blotting) 을 연속적으로 시행하였다. 첫번째 결과로 우선 HUVECS을 대상으로 TNF-α로 자극시킨 세포에 각각 cilostazol의 농도를 달리하여 4시간 동안 배양, TF PCR을 시행한 결과 cilostazol의 농도에 비례하여 TF발현이 억제됨을 관찰할 수 있었다. LPS로 자극시킨 단핵구에서도 위의 제대혈 세포와 같은 양상을 보였다. 공히 두 세포에서 24시간 세포 배양 후 실시한 Western blot analysis를 통하여 TF protein을 확인한 결과 cilostazol의 농도에 비례하여 TF protein이 억제됨을 알 수 있었다.

결론적으로 cilostazol은 HUVECS와 monocyte에서 TF-mRNA와 TF protein의 발현을 억제하는 기전을 갖는 것으로 생각된다.

핵심 되는 말; cilostazol, tissue factor(TF), monocytes, HUVECs