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Angiopoietin-1 이 interleukin-6 에 의해 유도된 내피 투과성에 미치는 영향에 관한 연구

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Study on the effects of angiopoietin-1 on interleukin-6–induced endothelial permeability

by

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**ABSTRACT**

**Introduction:** Diabetic retinopathy (DR) is a diabetic complication that occurs in patients with long-term diabetes. The pathological symptom of DR appears on the retina, which causes blood or fluids to leak out of the blood vessels. Vascular leakage makes it difficult for the image to focus on the retina. Several factors, vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), connective tissue growth factor, and intercellular adhesion molecule-1 (ICAM-1), causes abnormal blood vessels in diabetic retinopathy. One of these well-known factors, VEGF causes breakdown of the blood retinal barrier and retinal neovascularization. Factors that up-regulate VEGF include hypoxia-inducible factor-1 (HIF-1), hyperglycemia, AGEs, growth factors such as insulin, insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF), and pro-
inflammatory cytokines. Among these cytokines, interleukin-6 (IL-6) is a typical pro-inflammatory cytokine that affects up-regulation of VEGF. Meanwhile, angiopoietin-1 (Ang-1), a protein that affects vascular maturation and stability reduces blood vessel leakage by diabetic retinopathy, but its mechanism related to pro-inflammatory cytokine-induced vascular leakage is not clear. Therefore, I tried to identify how Ang-1 inhibits IL-6 through this study.

**Methods:** IL-6 or (and) Ang-1 were treated with human umbilical vein endothelial cells. After that, I confirmed ZO-1, Occludin, p-STAT3, p-p38 proteins expression levels using western blotting analysis. In addition, I analyzed the endothelial permeability of HUVEC treated with IL-6 or (and) Ang-1 by trans-endothelial permeability assay.

**Results:** IL-6 increased STAT3 expression and decreased tight junctional proteins, ZO-1 and Occludin, expression in endothelial cells. This phenomenon caused endothelial
permeability. However, treatment of Ang-1 reduced IL-6-induced STAT3 activation, decreased endothelial permeability and, increased ZO-1 and Occludin expression. p38, which inhibits STAT3 and is activated by Ang-1, reduced IL-6-induced STAT3 activation in endothelial cells.

Conclusions: These results suggest that Ang-1 mediates p38 activation and plays a critical role in inhibiting increased endothelial permeability due to STAT3 induced by IL-6. Therefore, the mechanism by which Ang-1 inhibits IL-6-induced STAT3 is important for the development of a therapeutic agent for diabetic retinopathy.

Keywords: Diabetic retinopathy, Interleukin-6, Angiopoietin-1, vascular permeability, angiogenesis

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LIST OF ABBREVIATIONS

Ang-1: Angiopoietin-1

DR: Diabetic retinopathy

HUVEC: Human umbilical vascular endothelial cell

IL-6: Interleukin-6

STAT3: Signal transducer and activator of transcription 3

VEGF: Vascular endothelial growth factor

ZO-1: Zonula occludens-1
INTRODUCTION

Diabetic retinopathy (DR) is a complication that occurs in more than 80% of diabetic patients for more than 20 years. In diabetic retinopathy, blood and fluid leak from retinal blood vessels, abnormal angiogenesis occurs, and image of the object to be viewed is not focused on the retina. If the illness gets worse, it will lead to blindness. It is well known that the cause of this disease is the up-regulation of vascular endothelial growth factor (VEGF), which breaks down the blood-retinal barrier and forms retinal neovascularization [1]. In addition, it is well known that the increase of various pro-inflammatory cytokines up-regulates VEGF in diabetic situations [1, 2]. Especially, interleukin-6 (IL-6) [2], one of the pro-inflammatory cytokines, induced signal transducer and activator of transcription 3 (STAT3) activation causes vascular inflammation that augments endothelial permeability and vascular leakage in the diabetic retina [3-5]. This cytokine is released from stromal cells around the endothelial cells and affects diabetes [6]. Increased endothelial permeability and vascular leakage are due to down-regulation of the tight
junctional proteins expression associated with endothelial cells [3]. These facts mean that activation of IL-6-induced STAT3 increases endothelial permeability and up-regulates VEGF, thereby inhibiting the expression of tight junctional proteins in diabetic retinopathy [3].

Meanwhile, angiopoietin-1 (Ang-1), one of the angiopoietin family, prevents diabetic retinopathy [7]. This protein has a critical role in vascular maturation, adhesion, migration, and survival [8]. Ang-1 up-regulates tight junctional proteins [9] that connect retinal microvascular endothelial cells [10], which are reduced in the diabetic retina [8, 11, 12]. Up-regulation of tight junctional proteins inhibits blood vessel leakage, hemorrhage, and inflammation of vessels and then induces retinal revascularization [8, 11, 12]. And, Ang-1 up-regulates p38 which is MAPK (mitogen-activated protein kinase). p38, stress kinase, acts as a diverse transcriptional regulator involved in cell proliferation, pro-survival, and stress-response pathway [13, 14]. Especially, STAT3 is inhibited by p38 [14].

Nevertheless, it is unclear whether Ang-1 prevents the reduction of tight junctional proteins expression by IL-6-
induced STAT3 activation in diabetic retina. Therefore, I thought that Ang-1 could directly inhibit IL-6-induced STAT3 activation to prevent endothelial permeability.

In this study, I demonstrated that p38 activation by Ang-1 inhibited the reduction of tight junctional proteins and increase of endothelial permeability due to IL-6-induced STAT3.
MATERIALS AND METHODS

1. Cell culture

Human umbilical vein endothelial cells (HUVEC, Lonza, Switzerland) were cultured in gelatin-coated (Welgene, Korea) 60mm petri dishes and 24 trans-well plates (SPL, Korea). Cells were maintained in M199 medium with 10% fetal bovine serum (FBS) and were cultured at 37°C in an incubator with a humidified atmosphere of 5% CO₂. Cells were changed in M199 medium with 1% FBS for starvation.

2. Western blotting analysis

HUVECs that treated interleukin-6 (Peprotech, Inc., USA, 20 ng/mL) or (and) angiopoietin-1 (Ang-1, R&D systems, Inc., USA, 300 ng/mL) were lysed in a lysis buffer that containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail. The cell lysates were centrifuged to separate the supernatant to be used for the experiment and the debris to be removed. Protein lysates were separated on 7% and 10% SDS-PAGE gels and were transferred to
nitrocellulose membranes. The membranes were incubated with primary antibodies (1:1,000) at 4°C overnight. Primary antibodies (Abs) were as following; anti-phospho-STAT3, anti-STAT3, anti-phospho-ERK, anti-ERK, anti-phospho-AKT, anti-AKT, anti-phospho-p38, anti-p38, anti-ZO-1 (Cell signaling technology, Inc., USA), anti-Occludin (Life technologies, USA) and anti-β-actin (Sigma-Aldrich Inc., Germany) Abs. The membranes were incubated secondary Abs (1:5,000) that were mouse and rabbit at room temperature for 1 hour. Thereafter, the membranes were incubated with an enhanced chemiluminescence substrate, and a pico chemiluminescence substrate (Pierce, IL, USA) and exposed to film. And, the graph of western blots was quantified by image J program.

3. Trans-endothelial permeability assay

HUVECs were cultured on trans-well plates (Corning, Inc., 12 inserts, 24 well plate, 0.4 μm pore size) for 3 days after seeding. Then, IL-6 (20 ng/mL) or (and) Ang-1 (300 ng/mL) were treated for 48 hours. To evaluate trans-endothelial permeability, Evans blue dye (Sigma-Aldrich, Inc., Germany)
containing bovine serum albumin was put in the upper chamber. After 5 minutes, the value of optical density of the medium in the bottom chamber was measured in a spectrophotometry (Infinite M200PRO, Tecan, Switzerland).

4. Statistical analysis

The data analyses performed using Prism software 5.0 (GraphPad Software, Inc., San Diego, CA, USA), and two-tailed Student’s t-test assuming unequal variances. A value of $p < 0.05$ was considered statistically significant. The data and figures are presented as mean ± standard deviation (SD).
RESULTS

Angiopoietin–1 inhibits IL–6–induced STAT3 activation in HUVEC

To confirm that effects of IL–6–induced STAT3 activation by Ang–1, the phosphorylation of STAT3 was analyzed by western blotting assay. Prior to the experiment, I set the concentrations of IL–6 and Ang–1 to 20, 300 ng/ml respectively (Fig. 1A–B). The treatment time referred to the known time [3]. The increased expression levels of phospho–STAT3 by IL–6 was verified in HUVEC. However, when IL–6 and Ang–1 was co–treated, the expression level of phospho–STAT3 was decreased (Fig. 1C–D). This result means that Ang–1 inhibits STAT3 activation induced by IL–6.
Figure 1. Effect of Ang-1 in the change of IL-6–induced STAT3 expression levels.

(A) IL-6 dose determined by western blotting assay as indicated concentration (20 ng/mL). (B) Ang-1 dose determined by western blotting assay as indicated concentration (300 ng/mL). (C) Expression levels of phospho-STAT3 analyzed by western blotting assay. IL-6 or (and) Ang-1 were treated for 30 minutes. The concentration of IL-6
and Ang−1 were 20 ng/mL and 300 ng/mL respectively. (D) Results of (C) quantified. *denotes $p < 0.05$ by Student’s $t$-test (n=3).
Angiopoietin-1 reduces endothelial permeability by IL-6-induced STAT3 activation

I examined the effect of Ang-1 in endothelial permeability by IL-6-induced STAT3. Because increased IL-6-induced STAT3 activation results in endothelial permeability. And, I already showed inhibition of IL-6-induced STAT3 activation by Ang-1 at the protein levels (Fig. 1). IL-6 increased endothelial permeability in HUVEC (Fig. 2A). However, in the condition of combination treatment with IL-6 and Ang-1, permeability in HUVEC was improved (Fig. 2A). Thus, I found that Ang-1 was involved in the reduction of endothelial permeability by STAT3 activation induced by IL-6.
Figure 2. Effect of Ang–1 on endothelial permeability by the IL–6–STAT3 pathway.

(A) The endothelial permeability of HUVEC was measured by trans–endothelial permeability assay. HUVEC was cultured in trans–well inserts for 72 h. After 72 h, IL–6 (20 ng/mL) or (and) Ang–1 (300 ng/mL) were treated for 48 h. * denotes p < 0.05 by Student’s t–test (n=3).
Angiopoietin–1 recovers reduction of tight junctional proteins by IL–6

IL–6 has a negative effect on expression of tight junctional proteins that keep a tight connection with endothelial cells. Also, increased expression of tight junctional proteins means that formation of mature vessels. Therefore, I examined the effects of Ang–1 on the decrease of tight junctional proteins by IL–6. The expression of tight junctional proteins (ZO–1 and Occludin) was analyzed by western blotting assay (Fig. 3A–B). As a result, reduced expression of ZO–1 and Occludin in IL–6 was recovered by Ang–1 (Fig. 3 A–B). That was because Ang–1 increased ZO–1 and Occludin expression levels which decreased by IL–6.
Figure 3. Effects of Ang-1 on tight junctional proteins reduced by IL-6.

(A) Expression levels of ZO-1 and Occludin were analyzed by western blotting assay. IL-6 or (and) Ang-1 were treated for 48h. The concentration of IL-6 and Ang-1 were 20 ng/mL and 300 ng/mL respectively. (B) Results of (A) was quantified. *denotes $p < 0.05$ by Student’s *t*-test (n=3).
Angiopoietin-1 mediated activation of p38 inhibits STAT3 activation

To identify mechanisms that Ang-1 inhibited expression of IL-6 induced STAT3 activation, I focused on activation of p38. p38 was well known that down-stream of Ang-1 and that directly inhibited activation of STAT3 in various types of cells. Therefore, I examined the effects of p38 activation involved with STAT3 activation by using p38 inhibitor (SB203580). Before the experiment, the time of IL-6, Ang-1, and p38 inhibitor and the concentration of p38 inhibitor was determined (Fig. 4A–C). In effects of preceding, Ang-1 inhibited STAT3 activation that was up-regulated by IL-6. However, Ang-1 had no effects on STAT3 activation by IL-6 with p38 inhibitor (Fig. 4D). Through this experiment, I verified that Ang-1 inhibited IL-6 induced STAT3 activation by p38 activation.
Figure 4. Expression of phospho–p38 by Ang–1 reduces STAT3 activation by IL–6.

(A–C) Determination of the time for Ang–1 and IL–6 (5min) and the concentration of p38 inhibitor (5 μM). (A) Ang–1 treated for 5, 15, and 30 minutes. Expression levels of phospho–p38 analyzed by western blotting assay. (B) IL–6 treated for 5, 15, and 30 minutes. Expression levels of phospho–STAT3 analyzed by western blotting assay. (C) p38 inhibitor (SB203580) treated for 1, 5, 10 μM. Expression levels of phospho–p38 analyzed by western blotting assay. (D) Expression levels of phospho–STAT3 and phospho–p38 analyzed by western blotting assay.
Angiopoietin-1 mediated activation of p38 inhibits IL-6-induced endothelial permeability

Following confirmation of the proteins expression level, I performed the endothelial permeability assay to identify the effect of p38 on endothelial permeability. In the absence of p38 inhibitor treatment, it was confirmed that the increase in IL-6-induced endothelial permeability was decreased by Ang-1 treatment (Fig. 5A). However, when treated with the p38 inhibitor, Ang-1 did not prevent endothelial permeability by IL-6 (Fig. 5A). This result suggests that Ang-1-activated p38 inhibits endothelial permeability by the IL-6-STAT3 pathway.
Figure 5. Effect of Ang−1 mediated p38 on endothelial permeability by IL−6−STAT3 pathway.

(A) Permeability of HUVEC was performed by trans−endothelial permeability assay. HUVEC was cultured in trans−well inserts for 72h. After 72h, p38 inhibitor (SB203580) (5 μM) was treated 1h. After that, IL−6 (20 ng/mL) and Ang−1 (300 ng/mL) treated for 48h. * denotes p < 0.05 by Student’ s t−test (n=5). n.s is not significant.
DISCUSSION

DR is one of the diabetic complications that appear in the retina of patients with long-term diabetes. The problem with this disease is that the blood-retinal barrier is destroyed and retinal neovascularization occurs [1]. It is well known that the cause of this disease is up-regulation of VEGF [1]. VEGF is up-regulated by a variety of factors. Especially, IL-6, one of the pro-inflammatory cytokines, has an effect on up-regulation of VEGF [2] and increases both IL-6 and VEGF in diabetic condition [4, 5]. IL-6 activates STAT3, a transcriptional factor, and causes diabetic retinopathy.

The increase of endothelial permeability means that decrease of tight junctional proteins expression that keeps each connection with endothelial cells [3]. Meanwhile, Ang-1, the protein of angiopoietin family, inhibits diabetic retinopathy by preventing or recovering endothelial permeability [7]. However, the mechanism by Ang-1 is not clear perfectly for diabetic retinopathy. Thus, I have investigated the mechanism by which Ang-1 reduces the endothelial permeability of the diabetic condition.
In endothelial cells, microglia-derived-IL-6 induced STAT3 activation decreases expression of tight junctional proteins, such as ZO-1 and Occludin, and then causes vascular leakage of the retina. This phenomenon is implications for diabetic retinopathy [3]. In addition, the Ang-1–Tie2 pathway in which Ang-1 binds to Tie2 has an important role in reducing vascular leakage [15, 16] by increasing expression of tight junctional proteins (Fig. 3).

I focused on the phenomena that STAT3 induced by IL-6 in the diabetic condition increases endothelial permeability and that the tight junctional proteins are increased by Ang-1. Therefore, I thought the effect of Ang-1 as an inhibitor of the IL-6–STAT3 pathway. For this reason, I hypothesized that Ang-1 would directly inhibit IL-6 induced STAT3 activation.

In the present study, I demonstrated that IL-6–induced STAT3 activation was reduced by Ang-1 in protein levels and endothelial permeability (Fig. 1, Fig. 2). And I confirmed that activation of p38, the down-stream of Ang-1, inhibited the endothelial STAT3 activation (Fig. 4) and endothelial permeability (Fig. 5).
However, reduction of STAT3 activation by p38 activation has still proven to be controversial. Because some investigation reports that p38 activation activates STAT3 [17]. Nevertheless, several investigations and my results show the reduction of STAT3 activation by p38 activation [13, 14] (Fig. 4D). Therefore, it is necessary to further study the interaction between p38 and STAT3 activation in other conditions.

In this study, I demonstrated that the activation of p38 by Ang-1 directly inhibits increased endothelial permeability due to STAT3 induced by IL-6. Therefore, through p38 activation, Ang-1 inhibits IL-6-induced endothelial STAT3 activation.

Limitations of this study are that my results have not identified in the diabetic mouse model. Although I hypothesized that Ang-1-activated p38 would reduce the expression of IL-6-induced STAT3 phosphorylation, IL-6 increased the expression of p38 phosphorylation. However, it is not clear that IL-6 increased p38 phosphorylation yet. Therefore, it needs to be identified exactly whether IL-6 affects p38 phosphorylation.

In conclusion, I found that p38 activation by Ang-1 reduced IL-6-induced endothelial STAT3 activation in diabetic condition. Therefore, Ang-1 and p38 activation can be critical
therapeutic medication for reducing endothelial permeability by the IL-6–STAT3 pathway in diabetic retina.
REFERENCES


국문 초록

서론: 당뇨망막병증은 오랜 기간 당뇨에 걸린 환자에게서 나타나는 합병증으로 병적인 증상이 눈의 망막에 나타났을 때 생기는 질환이다. 즉, 망막에 있는 혈관에서 혈액이나 체액의 누수로 인해 상이 망막에 밝히는 것을 방해한다. 당뇨망막병증에서 비정상적인 혈관 생성을 유발하는 여러 인자들은 혈관 내피세포 성장 인자인 VEGF 를 비롯하여, 종양 괴사 인자, 연결 조직 성장 인자, 그리고 세포간 부착 분자-1 등이 있다. 이 인자들 중의 하나인 VEGF 의 증가는 혈액망막방벽의 손상과 망막 혈관의 신생을 일으킨다. 이 VEGF 를 증가시키는 인자들은 저산소 유도 인자-1, 고혈당증, 최종당화산물과 인슐린, 인슐린 유산소성장 인자-1, 섬유 모세포 성장 인자, 혈소판 유래 성장 인자와 같은 성장인자, 그리고 염증성 사이토카인이 있다. 여러 염증성 사이토카인들 중 하나인 IL-6 는 VEGF 를 증가시키는 대표적인 사이토카인으로 잘 알려져 있다. 한편, Ang-1 은 혈관의 성숙과 안정화에 관여하는 단백질로 당뇨 망막 병증에 의한 혈관 누수를 막아준다고 알려져 있지만, 염증성 사이토카인에 의한 혈관 누수를 막아주는 자세한 기전에 대해서는 알려져 있지 않다. 따라서 본 연구를 통해, Ang-1 이 어떤 기전을 통해 IL-6 기전을 막는지 찾고 이 때 어떤 작용이 일어나는지 확인해 보고자 하였다.
방법: 당뇨상황에서 IL-6 가 증가되기 때문에 인간 절대 정맥혈관내피세포에 IL-6 를 처리하였다. 또한 혈관내피세포에 IL-6 나(와) Ang-1 을 처리하고, 이로 인해 변화가 일어나는 단백질의 발현량을 western blotting 방법으로 확인하였다. 혈관내피세포의 누수를 확인하는 trans endothelial permeability 방법을 통해 IL-6 나(와) Ang-1 으로 인한 누수 정도를 비교하였다.

결과: 혈관내피세포에 IL-6 를 처리하였을 때는 IL-6 에 의해 증가되는 STAT3 단백질의 발현은 증가하고, 혈관내피세포의 접합 복합체 단백질의 발현량을 줬다. 이로 인해,혈관내피세포의 누수가 증가하였다. 하지만 Ang-1 을 IL-6 와 함께 처리한 경우에는 STAT3 단백질의 발현은 감소하였고, 혈관내피세포의 접합 복합체 단백질의 발현은 증가하였다. 또한 혈관내피세포의 누수도 감소하는 것을 관찰할 수 있었다. 한편, 여러 세포에서 STAT3 단백질을 억제하는 것으로 알려진 p38 이 Ang-1 에 의해 활성화되며 혈관내피세포에서도 IL-6 에 의해 증가된 STAT3 단백질을 억제하는 현상을 관찰하였다.

결론: 당뇨망막병증에서 망막에 있는 혈관내피세포는 IL-6-STAT3 기전에 의해 혈관 내피 투과성이 증가되지만 Ang-1 에 의해 억제되어 혈관 내피 투과성의 증가를 막는다. 이는 Ang-1 에 의해 활성화된 p38 이 STAT3 를 억제하는 기전 때문이다. 따라서, 당뇨 상황에서 IL-6 에 의해 유도되는 혈관 내피 투과성의 증가를 막는데 있어서 Ang-1 과 p38
의 활성화가 중요하고 치료제 개발에 있어서도 중요한 요소라 생각한다.

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주요어 : 당뇨망막병증, Interleukin-6, Angiopoietin-1, 혈관 누수, 혈관 신생
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