VEGF と VEGF 受容体を標的とした難治性川崎病の治療に関する基礎的研究

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研究発表　（学会発表）

（1）Higashi K, Ebata R, Honda T, Yasukawa K, Hamada H, Terai M. In Vitro Endothelial Cell Dysfunction Induced By Serum From Patients With Kawasaki Disease Is Modulated By P38

Mitogen Activated Protein Kinase. 78th American Heart Association Scientific Sessions 2005, Dallas, Nov 12-16, 2005. (Circulation 112(17): Suppl II-476, 2005).

（2）Higashi, K., Ebata, R., Honda, T., Yasukawa, K., Tohyama, T., Jibiki, T., Hamada, H., Kanazawa, M., Terai, M. Impairment of serum angiogenic activity in acute Kawasaki disease. 8th Int Kawasaki Disease Symposium., San Diego Feb17-20, 2005.

（3）東浩二　浜田洋通　江畑亮太　遠山貴子　本田隆文　安川久美　寺井 勝: p38mitogen-activated protein kinase 阻害剤による治療の可能性,　日本川崎病研究会シンポジウム 2006 年 10 月　大阪

研究成果概要

免疫グロブリン治療が無効な川崎病難治例では冠動脈瘤が高率に発生し、難治性川崎病に対する新たな治療法の確立は社会的ニーズが高い。現在までの多くの研究成果から、難治性川崎病の病態にVEGF・VEGF受容体系が深く関わっていることが判明している。本研究では、VEGFやVEGF受容体を標的とした新たな抗炎症療法の可能性を解析する。本年度は、インビトロアッセイ系を用いて、川崎病で多量に産生されるVEGFが本当に血管透過性をもたらすのかを検証した。

川崎病患者血清では、血中VEGFは健常対照に比べ増加していた。また、これら患者血清を用いて、臍帯静脈血管内皮細胞の培養系に５％濃度の血清を添加し、培養臍帯静脈血管内皮細胞の血管透過性をインビトロで検討した。まず、川崎病の治療薬として用いられている、免疫グロブリンやアスピリンそのものは培養臍帯静脈血管内皮細胞の血管透過性に影響は与えなかった。

次に、患者血清について検討した。治療前川崎病患者血清は、健常対照者血清に比べて臍帯静脈血管内皮細胞の血管透過性機能には差が認められなかった。次に、免疫グロブリン治療後の患者血清を用いて検討したところ、免疫グロブリン治療により反応のあった患者群の血清には培養臍帯静脈血管内皮細胞の血管透過性機能には影響を与えなかったが、免疫グロブリンに抵抗を示しその後冠動脈異常を来たした抵抗例の血清を用いると、培養臍帯静脈血管内皮細胞の血管透過性が有意に増加した。また、VEGF受容体の抗体およびVEGFリガンドの生理活性抑制因子である可溶性 VEGFR-1 のいずれの前処理においても免疫グロブリン抵抗例の血清による血管透過性機能の亢進を抑制することができた。

以上より、患者血清にはVEGFが含まれており、治療抵抗例の血管透過性に重要な役割を果たしていることが推測される。

そこで、患者血清がもたらす培養臍帯静脈血管内皮細胞の血管透過性を阻害する薬物を探索した。VEGF受容体の下流にあるp38 MAPKの役割に着目した。P38 MAPK 阻害薬（SB203580）をアッセイ系に前処置して検討した結果、免疫グロブリン治療抵抗例の血清にみられた血管透過性亢進作用は阻害された。また、その他の ERK 阻害剤ではこのような効果は認めず、川崎病血清に存在する血管透過性作用は、p38-MAPK を介している可能性が示唆された。p38MAPK 阻害剤の治療薬としての可能性をさらに検討することも重要と考える。

In vitro endothelial cell dysfunction induced by serum from patients with refractory Kawasaki disease is modulated by p38 mitogen activated protein kinase

***Background*:** The p38 mitogen-activated protein kinase (MAPK) signaling pathway plays an important role in the pathogenesis of inflammatory diseases. p38 MAPK can be activated by proinflammatory cytokines and vascular endothelial growth factor. However, nothing is known about the role of p38 MAPK in acute Kawasaki disease (KD) where these inflammatory mediators are elevated. ***Methods and Results*:** p38 MAPK was strongly activated in peripheral blood mononuclear cells from patients during acute KD inflammation and down-regulated in the convalescent phase of KD. Additionally, incubation of human umbilical vein endothelial cells (HUVEC) with KD sera before high-dose intravenous immune globulin (IVIG) induced a rapid increase in p38 MAPK phosphorylation. This activation of p38 MAPK was further enhanced when HUVEC were treated with post-IVIG sera from patients who failed to respond to initial IVIG and later developed coronary aneurysms (IVIG failure), but was down-regulated when treated with post-IVIG sera from patients who responded to initial IVIG and had normal coronaries (IVIG responder). Compared with sera from IVIG responders or febrile control, sera from IVIG failure induced not only the increase in HUVEC permeability but also the decrease in the tube forming activity of HUVEC. The p38 MAPK inhibitor blocked IVIG failure sera-induced vascular permeability. The same inhibitor restored the tube forming activity of HUVEC induced by sera from IVIG failure, a finding that was accompanied by enhancement of Erk1/2 MAPK activation. ***Conclusions*:** The findings suggest that *in vitro* endothelial cell dysfunction induced by serum from patients with refractory KD is modulated by p38 MAPK. (250 words)

Introduction

Kawasaki disease (KD), a systemic vasculitis, is characterized by immune activation associated with increased production of inflammatory cytokines and chemokines.[1-3] High-dose intravenous immune globulin (IVIG) is effective in the rapid resolution of acute KD inflammation and reducing the incidence of coronary aneurysms.[4-6] However, approximately 10 to 20% of patients have persistent or recurrent fevers after IVIG completion and are considered to have a higher risk of developing coronary aneurysms.[2-10] The management of these IVIG-resistant patients has not been established.

Elevated circulating levels of inflammatory mediators such as pro-inflammatory cytokines and chemokines decrease after IVIG therapy in patients who respond to IVIG and have normal coronaries.[11-17] Conversely, patients who fail to respond to IVIG and develop coronary aneurysms often have the persistent production of inflammatory cytokines by circulating and tissue immune cells.[18-20] Vascular endothelial growth factor (VEGF) is another important molecule in the development of coronary aneurysms after KD.[20] Many studies have suggested the association of VEGF with the development of coronary aneurysms.[20-23] These findings suggest that inflammatory mediators play an important role in the progression of coronary vasculitis in KD, although the precise mechanisms have not been elucidated.

Recently, the p38 mitogen-activated protein kinase (MAPK) signaling pathway is considered to play an important role in the pathogenesis of inflammatory diseases.[24] The p38 MAPK is mainly activated by tumor necrosis factor (TNF)-alpha and interleukin-1.[24,25] Recent studies have demonstrated that p38 MAPK is also activated by several growth factors including VEGF.[26] The activation of p38 MAPK is involved

in endothelial cell responses, including permeability and cell survival.[26-28] However, nothing is known about the role of p38 MAPK in acute KD where a variety of inflammatory mediators are elevated. We hypothesized that p38 MAPK is an important signal molecule modulating endothelial dysfunction of acute KD.

To test this hypothesis, we studied the activation of p38 MAPK in peripheral blood mononuclear cells (PBMCs) from KD patients. In addition, we examined KD serum-induced *in vitro* kinetics of human umbilical vein endothelial cells (HUVEC) to seek the role of p38 MAPK in endothelial cell dysfunction of KD.

Methods:

Reagents

Recombinant human VEGF and VEGF R1 (Flt-1)/Fc Chimera (soluble VEGFR1), were obtained from R & D Systems. VEGF Receptor 2 inhibitor I (VEGFR2 inhibitor I), Erk1/2 (extracellular signal-regulated kinase kinase1/2) kinase (MEK) inhibitor (PD98059) and p38 MAPK inhibitor (SB203580) were obtained from Calbiochem. Rabbit antibodies against phospho-p38 MAPK (Thr-180/Tyr-182), p38 MAPK, phospho-Erk1/2 (phospho-p44/42 (Thr202/Tyr204)), and Erk1/2 (p44/42 MAPK) were purchased from Cell Signaling Technology.

Patients and preparation of blood samples

In experiments using PBMCs, we studied paired heparinized blood samples from 4 patients (37 ± 17 month old at onset) who developed KD between December 2004 and March 2005. Paired samples were obtained on day 4 to 7 (5.0 ± 0.7) before KD treatment and on day 12 to 22 (15.0 ± 2.4) in the convalescent stage after normalization

of C-reactive protein. All the patients were treated with IVIG (2g/kg over 2 days) and oral aspirin (30 mg/kg/day), and became afebrile immediately after completion of 2g/kg IVIG.

In experiments using serum samples, we studied paired serum samples from 18 KD patients (Table 1), in whom paired samples before and just after initial IVIG (2g/kg over 1 to 5 days) were available between 1999 and 2004. Ten-paired samples were obtained from 10 patients (34 $\pm$ 8 months old at KD onset) who responded to treatment with initial IVIG and did not have coronary artery abnormalities (IVIG responder). We randomly selected these 10 patients from IVIG-responsive patients during the same period. The remaining 8-paired samples were obtained from the 8 patients (47 $\pm$ 9 months old at KD onset) who failed to respond to initial IVIG and later developed coronary aneurysms (> 4mm) despite of additional therapy (IVIG failure). Between 1999 and 2004, 22 IVIG failure patients were referred to our hospital, but paired samples were available only in these 8 patients, six of which were initially treated outside of our hospital.

Sixteen of the 18 patients also received oral aspirin (30 mg/kg/day) and the remaining two patients received intravenous heparin infusion (10 units/kg/hr) instead of aspirin because of elevation in serum transaminases. Samples from patients who received steroids along with initial IVIG were not selected in this study. Thus, all the samples for this study did not contain steroids.

For study reference, serum samples were collected from 10 healthy young children (46 $\pm$ 9 months) and 8 febrile patients (46 $\pm$ 11 months) with acute infection due to group A streptococci and adeno or influenza virus. All aliquot samples were frozen until the use. The study was approved by the institutional review committee of Chiba

University School of Medicine. Written informed consent for the study was obtained from the parents of patients and controls.

Endothelial cell culture

HUVEC were obtained from Cambrex and were cultured in endothelial cell basal medium (EBM-2, Cambrex) maintained at 37°C in 5% CO2 and supplemented with 5% fetal bovine serum, penicillin/streptomycin, and endothelial cell growth supplement (SingleQuots, Cambrex). The cells were used in experiments between passage number 2 and 4.

Western blot analysis

Western blot experiments were performed as described earlier.[11] Briefly, subconfluent cultures of HUVEC (passage 3-4) were serum-starved for 4 hours and then stimulated with 5% serum from either a patient or a control for 15 min or 18 hours. In some experiments, HUVEC were pretreated with 10μM SB203580 for 30 min before stimulation with 5% serum. HUVEC were washed twice with ice-cold PBS, and total cell lysates were prepared by scraping the cells in lysis buffer (50 mM Tris-HCl, 0.1 mM EDTA, 1% (v/v) Nonidet P-40, 0.1% SDS, 0.1% deoxycholic acid, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mg/L aprotinin, and 10 mg/L leupeptin). After removal of insoluble material, soluble supernatant was treated with SDS and boiled. For Western blot analysis of PBMCs, cells were isolated by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences).

Equal amounts of protein for each sample were loaded into 12.5 % polyacrylamide

gel (Daiichi Pure Chemicals), separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Bio-Rad). For immunoblotting, rabbit polyclonal antibodies were used with 1:1,000 dilution as described in manufacturer protocol.

Endothelial cell permeability assay

HUVEC were seeded at concentrations of 5 x 10^4 cells in 200μL of endothelial growth medium (EGM-2, Cambrex) onto Transwell polyester membranes (0.4-μm pore size; Corning) that had been precoated with fibronectin (20μg/cm^2; Sigma). Fully confluent HUVEC monolayers were cultured onto transwell membranes for 3-5 days. After removal of medium, HUVEC monolayers were incubated with EBM-2 for 2 hours, and then FITC-dextran (1 mg/mL, average molecular weight 40 000; Sigma) was added into the upper compartment of the transwell cultures, followed by stimulation for 2 hours with 5% serum from either a patient or a control. For inhibitory experiments, HUVEC were pretreated with 5μM SB203580 or 5μM PD98059 for 30 min before stimulation with 5% serum. The amount of FITC-dextran in the culture medium (50 μL) taken from the lower compartment was then determined with a fluorescence plate reader (SPECTRA MAX^R, Molecular Devices), using an excitation wavelength of 492nm, and detecting emission at 520nm. Activation of HUVEC monolayer permeability was quantified as clearance of FITC-dextran from upper compartment to lower compartment (after subtracting background fluorescence from all values) using the following equation. Permeability clearance = the amount of FITC-dextran in lower compartment / the amount of total FITC-dextran in both upper and lower compartments x 100. Each sample was examined in triplicate and the mean of the 3 values was used in the final data analysis.

Endothelial cell proliferation assay

HUVEC were grown in a 25 cm^2 tissue culture flask (Asahi Technoglass) until they were about 80% confluent. The cells were then trypsinized, washed, counted, and resuspended in EBM-2. HUVEC were plated in a 96-well cell culture plate (1.0×10^4 cells per well) in EBM-2 supplemented with 5% serum from either a patient or a control, and incubated at 37°C in 5% CO2 for 18 hours. Cells were washed with PBS and stained with 1.6 % Calcein AM (Molecular Probes) in Hank's balanced salt solution (Nikken Bio Medical) at 37°C for 90 minutes. Fluorescence of cells was measured in a fluorescence plate reader (SPECTRA MAX^R) at excitation/emission wavelengths of 485/530 nm. Each sample was examined in triplicate and the mean of the 3 values was used in the final data analysis.

Endothelial cell tube formation assay

Growth factor-reduced Matrigel (BD Biosciences) was placed in the well of a pre-chilled 48-well cell culture plate (Asahi Technoglass) and incubated at 37°C for 30 minutes to allow polymerization. HUVEC, at concentrations of 3×10^4 per well, were plated onto the growth factor-reduced Matrigel-coated wells, incubated at 37°C in 5%CO2 for 18 hours in the presence of 5% serum from either a patient or a control, and photographed using an inverted phase contrast photomicroscope (Leica Microsystems) and tube length was quantitatively analyzed by measurement of tube length. A mean of 4 different areas at a magnification of 40x, which covered 80 % area of each well in 48-well cell culture plate, was determined for each sample.

For inhibitory assays, cells were preincubated with each inhibitor for 30 minutes at

37°C, and then plated onto the growth factor-reduced Matrigel-coated wells, incubated at 37°C in 5% CO2 for 18 hours in the presence of 5% serum.

Statistics

The serum from the same healthy adult volunteer was always used as an internal control in the assay and defined as 100%. The percent increase or decrease in the HUVEC function relative to the internal control was calculated for each sample. All parameters were presented as mean ± standard error (SE). When the data followed a normal distribution, comparisons between two groups were performed using an unpaired Student's *t* test or a Welch's *t* test. If the data did not follow a normal distribution, then a Mann Whitney *U* test was used. P-values of < 0.05 were considered significant.

Results:

Activation of p38 MAPK in PBMCs from KD patients

First, we investigated whether or not p38 MAPK is activated in the acute stage of KD. For this purpose, we measured the p38 MAPK activity in PBMCs from 4 KD patients by detecting phosphorylated p38 MAPK using Western blot analysis (Figure 1). In all the 4 patients studied, p38 MAPK activation in PBMCs was strongly documented in the acute stage of illness before IVIG therapy and down-regulated in the convalescent phase of illness after IVIG therapy, suggesting the p38 MAPK activation in PBMCs from patients with acute inflammation of KD.

KD serum from IVIG failure enhances activation of p38 MAPK in HUVEC

It is difficult to investigate directly vascular endothelial cells of KD patients so that we examined the phosphorylated p38 MAPK in cultured HUVEC stimulated by serum from patients with acute KD. In this *in vitro* assay, we used 8-paired serum samples before (pre) and after (post) initial IVIG from 8 patients. Four of the 8 patients were IVIG responder, whereas the remaining 4 patients were IVIG failure. When HUVEC were stimulated for 15 min with sera, pre-IVIG sera from 6 patients (p2-6, p8) induced a rapid activation of p38MAPK in HUVEC compared with control serum (Figure 2). More importantly, this activation of p38 MAPK was further enhanced when HUVEC were treated with post-IVIG sera from IVIG failure (p6-8), but was down-regulated when treated with post-IVIG sera from IVIG responder (p1-3). Furthermore, the prolonged incubation of HUVEC for 18 hours with post-IVIG sera from IVIG failure (p5-7) induced the sustained and strong activation of p38 MAPK, whereas p38 MAPK was inactivated when HUVEC were incubated with post-IVIG sera from IVIG responder (p1,2,4). Thus, the difference in the activation of p38 MAPK was correlated with IVIG responsiveness when HUVEC were incubated for longer time with KD sera.

Serum from IVIG failure enhances HUVEC permeability

Next, we studied serum activity to promote HUVEC permeability, because KD is characterized by increased microvascular permeability. When stimulated with control serum, serum activity to promote HUVEC permeability was 107 $\pm$ 3 % of an internal control in healthy control and 89 $\pm$ 6 % in febrile control. The baseline serum activity to promote HUVEC permeability was similarly elevated in IVIG responder (n=10, 115 $\pm$ 9 %, p=0.04 vs. febrile control) and IVIG failure (n=8, 114 $\pm$ 9 %, p=0.04 vs. febrile control, p=0.91 vs. IVIG responder) compared with febrile control serum. When treated

with sera after initial IVIG (Figure 3A), its activity was further increased to 135 ± 6 % in IVIG failure (n=8, p=0.0006 vs. febrile control); it was higher than that in IVIG responder (n=10, 109 ± 7 %, p=0.03 vs. IVIG failure, p=0.04 vs. febrile control).

Inhibition of VEGF blocks HUVEC permeability induced by sera from IVIG failure

Next, we studied effects of inhibition of VEGF activity on HUVEC permeability induced by post-IVIG sera from IVIG failure, because VEGF plays a role in the vascular leakage of KD.[20,23] Preliminary experiments confirmed that external addition of VEGF increases permeability of HUVEC monolayers in a dose-dependent fashion (data not shown). The increase in HUVEC permeability induced by post-IVIG sera from 8 patients with IVIG failure was prevented by external addition of soluble VEGFR-1 (p=0.016) and VEGFR-2 inhibitor I (p=0.004), suggesting the involvement of serum VEGF in the control of the permeability of cultured HUVEC.

Serum from IVIG failure reduces tube forming activity of HUVEC

Additionally, we investigated serum activity to stimulate HUVEC tube formation. The conditions of the *in vitro* angiogenesis assay were adjusted so that HUVEC were capable of forming capillary-like tube structures in the well coated by growth factor-reduced Matrigel in the presence of healthy human serum, but failed in the absence of healthy control serum (Figure 3B-a). Under these experimental conditions, baseline serum from a patient with active Takayasu arteritis extensively promotes HUVEC tube formation, and its efficacy was 147% of internal control serum (Figure 3B-b). Tube forming activity of HUVEC in the presence of healthy (Figure 3B-c) and febrile control serum were 95 ± 2 % and 93 ± 5 % of internal control serum,

respectively. When treated with baseline sera before IVIG, tube forming activity of HUVEC was 88 ± 8 % in the presence of serum from IVIG responder (n=10, p=0.60 vs. febrile control), whereas it was 71 ± 5 % in the presence of serum from IVIG failure (n=8, p=0.005 vs. febrile control, p=0.09 vs. IVIG responder). When treated with KD sera after initial IVIG (Figure 3C), tube forming activity of HUVEC in the presence of serum from IVIG failure (68 ± 5 %) was still reduced compared with serum from febrile control (p=0.007) or IVIG responder (82 ± 5 %, p=0.10 vs. IVIG failure).

Proliferating activity of HUVEC in the KD sera after IVIG treatment

Capillary-like tube formation requires several biological activities, such as endothelial cell proliferation, cell migration, and cell-to-cell interaction. To investigate mechanisms of impairment of HUVEC tube formation induced by sera from IVIG failure, we additionally examined serum activity to proliferate HUVEC. The serum activity of healthy and febrile control was 95 ± 2 % and 119 ± 6 % of internal control, respectively. When treated with KD serum after initial IVIG (Figure 3D), serum from IVIG failure (n=8, 89 ± 5 %) was less active in stimulating HUVEC proliferation compared with serum from IVIG responder (n=10, 108 ± 4 %, p=0.008 vs. IVIG failure) or febrile control (p=0.002), but there was no significant difference in the serum activity between IVIG failure and healthy control (p=0.27).

p38 MAPK inhibitor restores KD serum-induced endothelial cell dysfunctions

Next, we investigated whether inhibition of p38MAPK activity may have a favorable effect on HUVEC kinetics induced by serum from IVIG failure. We first confirmed that pretreatment with p38MAPK inhibitor (SB203580) reduced the phosphorylated

p38MAPK in cultured HUVEC stimulated by serum from IVIG failure (data not shown).

We then investigated the effects of SB203580 or MEK1/2 inhibitor (PD98059) on abnormal HUVEC kinetics induced by post-IVIG serum in IVIG failure. External addition of SB203580, but not PD98059, significantly reduced the increase in HUVEC permeability induced by post-IVIG serum in IVIG failure (n=4) from 127 $\pm$ 5 % to 97 $\pm$ 9 % (p=0.027) (Figure 4A). Additionally, HUVEC tube forming activity induced by post-IVIG serum in IVIG failure (n=4) increased from 63 $\pm$ 2 % to 97 $\pm$ 2 % (p=0.002) by the addition of SB203580 (Figure 4B-C), suggesting a negative regulatory role of p38 MAPK under this assay system. The external addition of PD98059 or VEGF at 10ng/mL failed to show a significant rescue in the decreased tube forming activity of HUVEC induced by post-IVIG sera from IVIG failure.

Erk1/2 MAPK phophorylation in HUVEC upregulates by inhibition of p38 MAPK

We investigated effects of inhibition of p38 MAPK activity on phosphorylation of Erk1/2 MAPK to seek possible mechanisms by which p38 MAPK inhibitor restored the impairment of HUVEC tube formation induced by sera from IVIG failure. When HUVEC were stimulated for 15 min with serum from IVIG failure, the rapid increase in phophorylation of Erk1/2 MAPK was observed, but p38 MAPK inhibition did not affect phophorylation of Erk1/2 MAPK (Figure 5). However, when HUVEC were incubated for 18 hours with serum from IVIG failure, sustained p38 MAPK activation led to slight decrease in Erk1/2 MAPK activation, whereas addition of p38 MAPK inhibitor strongly enhanced Erk1/2 MAPK phosphorylation.

Discussion

The MAPK family mediates a wide variety of cellular functions in response to extracellular stimuli.[24] Three distinct MAPK pathways have been determined in mammalian cells, the Erk1/2, c-jun N-terminal kinase (JNKs), and p38. p38 MAPK is chiefly activated by the proinflammatory cytokines, growth factors and cell stress-inducing factors, and oxygen radicals.[29] Activation of p38 MAPK promotes synthesis of proinflammatory cytokines such as TNF-alpha, interleukin-1, -6, and -8,[30-32] all of which are upregulated in acute KD.[3,4] In addition, p38 MAPK controls the synthesis of other compounds, including chemokines, adhesion molecules, and other compounds involved in inflammation;[25] thus a strong link has been suggested between the p38 MAPK pathway and inflammation. Because inhibiting p38 MAPK suppresses the production and action of inflammatory cytokines, it is postulated that the use of p38 MAPK inhibitors in inflammatory diseases where these cytokines are elevated might be beneficial. Indeed, p38 MAPK inhibitors such as SB203580 have been demonstrated to be effective in animal models of rheumatoid arthritis.[33] Inhibitors are being developed with an aspect to treating other inflammatory diseases such as Crohn's disease.[34-36] However, the role of p38 MAPK in the pathogenesis of KD remains to be elucidated.

The present study is the first report to document the involvement of p38 MAPK in KD vasculitis. We found several new findings regarding p38 MAPK in the pathogenesis of KD. The first of our main findings was that p38 MAPK was strongly activated in PBMCs obtained from patients with acute KD. This finding suggests that acute inflammation due to KD lead to the activation of p38 MAPK in PBMCs. Histopathologic studies have shown that early coronary vascular lesions of KD are characterized by infiltration of large numbers of mononuclear cells.[11,18-20] It is reported

that p38 MAPK pathway involves in monocytic transendothelial migration,[37] and plays essential roles in the production of proinflammatory cytokines.[24] Indeed, various proinflammatory cytokines are produced by circulating and inflammatory mononuclear cells of patients with acute KD.[17-20]

The second of our main findings was that acute KD sera were found to induce the activation of p38 MAPK in cultured HUVEC. The activation of p38 MAPK in cultured HUVEC was correlated with IVIG responsiveness when HUVEC were incubated with sera after initial IVIG. More importantly, sera from patients developing refractory KD induced abnormal HUVEC kinetics. HUVEC incubated with post-IVIG sera from IVIG failure were hyperpermeable and failed to form capillary-like tube structures. p38 MAPK inhibitor reversed abnormal HUVEC kinetics induced by sera from IVIG failure.

The present study suggested that KD sera contain soluble factors promoting p38 MAPK activation in PBMCs and cultured endothelial cells. Although a variety of serum factors may be candidates promoting the increase in HUVEC permeability through p38 MAPK activation, we focused on the involvement of VEGF in this study. VEGF is known to increase the vascular permeability of microvessels to circulating macromolecules. Indeed, VEGF has been upregulated in circulating and inflammatory cells of KD patients.[20,21,23] We tested the effects of the external addition of soluble VEGFR-1 and VEGFR2 inhibitor I to examine the VEGF-induced effect, and found that VEGF is one of molecules promoting the increased vascular permeability *in vitro*. A recent study has shown that inhibition of p38 MAPK activity abrogated VEGF-induced vascular permeability *in vivo and in vitro*, suggesting the involvement of p38 MAPK in the regulation of vascular permeability.[38] In addition, several studies have suggested

that p38 MAPK is a key regulator of vascular permeability by VEGF.[27,38] Taken together with our findings, VEGF and p38 MAPK are involved in the control of HUVEC monolayer permeability.

Unexpectedly, KD sera inhibited the tube forming activity of HUVEC. Because KD sera included increased levels of free VEGF, we expected that KD sera had increased angiogenic activity. The precise mechanisms by which sera from IVIG failure reduce HUVEC tube formation are unknown. Since p38 MAPK inhibitor reversed tube forming activity of HUVEC, a negative role of p38 MAPK is at least suggested in the control of tube forming activity of endothelial cells induced by KD sera. A recent study indicates that inhibiting p38 MAPK enhances VEGF-induced angiogenesis *in vitro and in vivo*.[38] p38 MAPK inhibition has been found to accelerate bFGF-mediated angiognenesis *in vitro and in vivo*,[39] suggesting that p38 MAPK signaling is a more general anti-angiogenic mechanism. Also cross-talk between p38 and Erk may affect our experimental results. A recent study has demonstrated that sustained activation of p38 MAPK causes the decrease in Erk1/2 MAPK activity.[40] In this study, prolonged incubation of HUVEC with sera induced sustained p38 MAPK activation and slight decrease in Erk1/2 MAPK activation, but inhibition of p38 MAPK resulted in prolonged activation of Erk1/2 MAPK.

It is well known that aspirin has anti-angiogenic activity by blocking prostaglandin synthesis in endothelial cells through inhibition of cyclooxygenase.[41] Therefore, even small doses of aspirin may cause impairment of *in vitro* HUVEC tube formation promoted by human serum. To explore this possibility, we tested the difference in the serum activity to stimulate HUVEC tube formation between KD patients (n=10) treated with IVIG plus aspirin and those (n=10) treated with IVIG and heparin, and we found

that HUVEC tube formation promoted by serum was same throughout the illness between the groups (data not shown). Furthermore, additional experiments with the exogenous addition of acetylsalicylic (aspirin) or salicylic acid at various doses confirmed that a low concentration of acetylsalicylic or salicylic acid did not show significant inhibitory effects on HUVEC tube formation in the presence of human serum (data not shown). Finally, in patients with IVIG failure, sera before IVIG plus aspirin treatment strongly activated p38 MAPK and also affected HUVEC tube formation. With these results taken together, it was unlikely that the aspirin used for KD treatment caused impairment of tube forming activity of HUVEC in the presence of sera from IVIG failure.

In conclusion, we demonstrated the p38 MAPK activation in PBMCs from patients with acute KD inflammation. Serum from patients with refractory KD induced *in vitro* endothelial cell dysfunction, which was modulated by p38 MAPK. It is important to further elucidate the precise role of MAPK signaling pathway in the pathogenesis of KD vasculitis to develop a new molecular target for treating patients with refractory KD.

References

1. Kawasaki T. Acute febrile mucocutaneous syndrome with lymphoid involvement with specific desquamation of the fingers and toes in children: clinical observations of 50 cases (in Japanese). *Arerugi.* 1967;16:178–222.
2. Shulman ST, Rowley AH. Advances in Kawasaki disease. *Eur J Pediatr.* 2004;163:285-291.
3. Burns JC, Glode MP. Kawasaki syndrome. *Lancet.* 2004;364:533-544.
4. Newburger JW, Takahashi M, Gerber MA, et al. Diagnosis, treatment, and long-term management of Kawasaki disease. *Circulation.* 2004;110:2747-2771.
5. Furusho K, Kamiya T, Nakano H, et al. High-dose intravenous gammaglobulin for Kawasaki disease. *Lancet.* 1984;2:1055-1058.
6. Newburger JW, Takahashi M, Burns JC, et al. The treatment of Kawasaki syndrome with intravenous gamma globulin. *N Engl J Med.* 1986;315:341-347.
7. Tse SML, Silverman ED, MaCrindle BW, et al. Early treatment with intravenous immuneglobulin in patients with Kawasaki disease. *J Pediatr.* 2002;140:450-455.
8. Burns JC, Capparelli EV, Brown JA, et al. Intravenous gamma-globulin treatment and retreatment in Kawasaki disease. US/Canadian Kawasaki Syndrome Study Group. *Pediatr Infect Dis J.* 1998;17:1144-1148.
9. Wallace CA, French JW, Kahn SJ, et al. Initial intravenous gammaglobulin treatment failure in Kawasaki disease. *Pediatrics.* 2000;105:E78.
10. Kuijpers TW, Biezeveld M, Achterhuis A, et al. Longstanding obliterative panarteritis in Kawasaki disease: lack of cyclosporin A effect. *Pediatrics.* 2003;112:986-992.
11. Terai M, Jibiki T, Harada A, et al. Dramatic decrease of circulating levels of

monocyte chemoattractant protein-1 in Kawasaki disease after gammaglobulin treatment. *J. Leukocyte Biol.* 1999;65:566-572.

12. Maury CPJ, Salo E, Pelkonen P. Circulating interleukin-1 in patients with Kawasaki disease. *N Engl J Med.* 1988;319:1670.
13. Ueno Y, Takano N, Kanegane H, et al. The acute phase nature of interleukin 6: studies in Kawasaki disease and other febrile illnesses. *Clin Exp Immunol.* 1989;76:337-342.
14. Furukawa S, Matsubara T, Jujoh K, et al. Peripheral blood monocyte/macrophages and serum tumor necrosis factor in Kawasaki disease. 1988;48:247-251.
15. Lin CY, Lin CC, Hwang B, et al. Serial changes of serum interleukin-6, interleukin-8, and tumor necrosis factor-alpha among patients with Kawasaki disease. *J. Pediatr.* 1992;121:924.
16. Eberhard BA, Andersson U, Laxer RM, et al. Evaluation of the cytokine response in Kawasaki disease. *Pediatr Infect Dis J.* 1995;14:199 –203.
17. Abe J, Jibili T, Noma S, et al. Gene expression profiling of the effect of high-dose intravenous Ig in patients with Kawasaki disease. *J Immunol.* 2005;174:5837-5845.
18. Miura M, Garcia FL, Crawford SE, et al. Cell adhesion molecule expression in coronary artery aneurysms in acute Kawasaki disease. *Pediatr Infect Dis J.* 2004;23:931-936.
19. Gavin PJ, Crawford SE, Shulman ST, et al. Systemic arterial expression of matrix metalloproteinases 2 and 9 in acute Kawasaki disease. *Arterioscler Thromb Vasc Biol.* 2003;23:576-581.
20. Yasukawa K, Terai M, Shulman ST, et al. Systemic production of vascular endothelial growth factor and *fms*-like tyrosine kinase-1 receptor in acute Kawasaki

disease. *Circulation.* 2002;105:766 –769.

21. Maeno N, Takei S, Masuda K, et al. Increased serum levels of vascular endothelial growth factor in Kawasaki disease. *Pediatr. Res.* 1998;44:596-599.
22. Kariyasono H, Ohno T, Khajoee V, et al. Association of vascular endothelial growth factor (VEGF) and VEGF receptor gene polymorphisms with coronary artery lesions of Kawasaki disease. *Pediatr Res.* 2004 56:953-959.
23. Ohno T, Yuge T, Kariyazono H, et al. Serum hepatocyte growth factor combined with vascular endothelial growth factor as a predictive indicator for the occurrence of coronary artery lesions in Kawasaki disease. *Eur J Pediatr.* 2000:161:105-111.
24. Kumar S, Boehm J, Lee JC. p38 MAP kinases: key signaling molecules as therapeutic targets for inflammatory diseases. *Nat Rev Drug Discov.* 2003; 2: 717-726.
25. Herlaar E, Brown Z. p38 MAPK signaling cascades in inflammatory disease. Mol Med Today 1999; 5: 439-447.
26. Rousseau S, Houle F, Landry J, et al. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene.* 1997; 15: 2169-2177.
27. Clauss M, Sunderkotter C, Sveinbjornsson B, et al. A permissive role for tumor necrosis factor in vascular endothelial growth factor-induced vascular permeability. *Blood.* 2001; 97: 1321-1329.
28. Ono K, Han J. The p38 signal transduction pathway: activation and function. *Cell Signal.* 2000; 12: 1-13.
29. Raingeaud J, Gupta S, Rogers JS, et al. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual

phosphorylation on tyrosine and threonine. *J Biol Chem.* 1995;270:7420-7426.

30. Lee JC, Laydon JT, McDonnell PC, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature.* 1994;372:739-746.
31. Beyaert R, Cuenda A, Vanden Berghe W, et al. The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor. *EMBO J.* 1996;15:1914-1923.
32. Miyazawa K, Tetsuka T, Yoshida S, et al. Regulation of interleukin-1 beta-induced interleukin-6 gene expression in human fibroblast-like synoviocytes by p38 mitogen-activated protein kinase. *J Biol Chem.* 1998;273:24832-24838.
33. Smolen JS, Steiner G. Therapeutic strategies for rheumatoid arthritis. *Nat Rev Drug Discov.* 2003; 2: 473-488.
34. Zhu X, Lee HG, Raina AK, et al. The role of mitogen-activated protein kinase pathways in Alzheimer's disease. *Neurosignals.* 2002; 11: 270-281.
35. Lowenberg M, Peppelenbosch MP, and Hommes DW. Therapeutic modulation of signal transduction pathways. *Inflamm Bowel Dis.* 2004; 10: 52-57.
36. Badger AM, Griswold DE, Kapadia R, et al. Disease-modifying activity of SB 242235, a selective inhibitor of p38 mitogen-activated protein kinase, in rat adjuvant-induced arthritis. *Arthritis Rheum.* 2000;43:175-183.
37. Cambien B, Pomeranz M, Millet MA, et al. Signal transduction involved in MCP-1-mediated monocytic transendothelial migration. *Blood.* 2001; 97: 359-366.
38. Issbrucker K, Marti HH, Hippenstiel S, et al. p38 MAP kinase--a molecular switch between VEGF-induced angiogenesis and vascular hyperpermeability. *FASEB J.* 2003; 17: 262-264.
39. Matsumoto T, Turesson I, Book M, et al. p38 MAP kinase negatively regulates

endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis. J *Cell Biol.* 2002;156:149-160.

40. McMullen ME, Bryant PW, Glembotski CC, et al. Activation of p38 has opposing effects on the proliferation and migration of endothelial cells. *J Biol Chem* 2005;280: 20995-21003.

41. Tsujii M, Kawano S, Tsuji S, et al. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 1998;93:705-716.

Figure legends

Figure 1 **p38 MAPK in PBMCs from patients with KD.**

Western blot analysis of PBMCs lysates was performed with either phospho-p38 MAPK antibody (top) or p38 MAPK antibody (middle). Similar protein loading was detected as determined using an alpha-actin antibody (bottom). P=patient, A=acute stage before IVIG, C=convalescent stage.

Figure 2 **Sera from IVIG failure induce a rapid and prolonged phospholyration of p38 MAPK in cultured HUVEC.**

Subconfluent HUVEC were serum-starved for 4 hours and were incubated with sera from IVIG-responder (P.1-4) or -failure (P.5-8) for the indicated times. Prolonged incubation with sera induces sustained and strong activation of p38 MAPK when incubated with post-IVIG sera from 3 patients with IVIG failure (p5-7 in 18 h incubation), but p38 MAPK is inactivated when treated with post-IVIG sera from 3 IVIG responders (p1,2,4 in 18 h incubation). C1-2 indicates healthy control. Pre indicates serum before IVIG treatment. Post indicates serum just after initial IVIG treatment.

Figure 3 **Sera after initial IVIG in IVIG failure induced the increase in permeability and the decrease in tube formation of HUVEC**

A) HUVEC monolayers were starved in serum-free media for 2 hours, followed by stimulation for 2 hours with 5% serum either from patients after initial IVIG or control. HUVEC permeability stimulated by serum from an internal control was defined as 100%. Each bar shows the mean $\pm$ SE. * p=0.04, ** p=0.03, † p=0.0006.

B) *In vitro* angiogenesis assay. HUVEC, at concentrations of 3 x 10^4 per well, were plated onto the growth factor-reduced Matrigel-coated wells, incubated for 18 hours in the presence of 5% serum from either a patient or a control. HUVEC fail to migrate or join together in the absence of human serum (*a*). Tube forming activity of HUVEC in the presence of serum from a patient with active Takayasu arteritis (*b*), serum from a healthy child (*c*), post-initial IVIG serum from a patient with IVIG failure (*d*), and post-initial IVIG serum from a patient with IVIG responder (*e*).

C) Tube forming activity of HUVEC in the presence of 5% sera either from patients after initial IVIG treatment or control. HUVEC tube length stimulated by serum from an internal control was defined as 100%. Each bar shows the mean ± SE. * p=0.007, NS=not significant.

D) HUVEC were incubated with 5% serum from either patients after initial IVIG or control for 18 hours. Cells were stained with 1.6 % Calcein AM and proliferation rate was calculated as described in the method section. HUVEC proliferation stimulated by serum from an internal control was defined as 100%. Each bar shows the mean ± SE. * p=0.008, † p=0.002. NS=not significant.

Figure 4 **Inhibition of p38 MAPK restores the increased permeability of HUVEC and impaired tube forming activity induced by post-IVIG sera from IVIG failure.** DMSO was used to dissolve specific inhibitors, and tested alone as control. **A**) HUVEC were pre-incubated with 5μM SB203580 or 5μM PD98059 for 30 min followed by stimulation with 5% serum. Addition of SB203580, but not PD98059, resulted in a significant decrease in HUVEC permeability in the presence of sera after initial IVIG in IVIG failure. Each bar shows the mean ± SE from 6 healthy controls or 8 patients with

IVIG failure. * p=0.027. NS= not significant. **B**) HUVEC were pre-incubated with 10ng/mL VEGF, 10μM SB203580 or 10μM PD98059 for 30 min followed by stimulation with 5% serum. Addition of SB203580, but not PD98059 or VEGF, resulted in a significant rescue in HUVEC tube formation in the presence of sera after initial IVIG in IVIG failure. Each bar shows the mean ± SE from 4 healthy controls or 4 patients with IVIG failure. * p=0.002. NS= not significant. **C**) Representative photograph of *in vitro* angiogenesis promoted by serum from a healthy child (*a*) and post-IVIG serum from a patient with IVIG failure (*b*). Tube forming activity in the presence of serum from the same patient with IVIG failure is changed by the external addition of recombinant human VEGF at 10ng/mL (*c*), 10μM of PD98059 (*d*), and 10μM of SB203580 (*e*).

***Figure 5*. Effects of inhibition of p38 MAPK by pretreatment with SB203580 on Erk1/2 phosphorylation.**

Subconfluent HUVEC were serum-starved for 4 hours and were pretreated with 10μM SB203580 for 30 min, followed by stimulation with 5 % serum from a patient with IVIG failure for the indicated times.

Table.1 Demographic data of KD patients in HUVEC assay

	Control children		KD patients	
	Healthy	Febrile	IVIG responder group	IVIG failure group
Number (Male / Female)	10 (8 / 2)	8 (5 / 3)	10 (6 / 4)	8 (6 / 2)
Age at onset (month after birth)	46 ± 9	46 ± 11	34 ± 8	47 ± 9
Sample points (days after onset)				
Before IVIG			5.5 ± 0.3	4.6 ± 0.5
Just after initial IVIG			10.0 ± 0.4	9.9 ± 1.0
Initial IVIG (2g/kg)			10	8
ASA (30mg/kg/day)			10	6
Heparin (10U/kg/hr)			0	2
Steroids			0	0
Patients developed coronary aneurysms			0	8

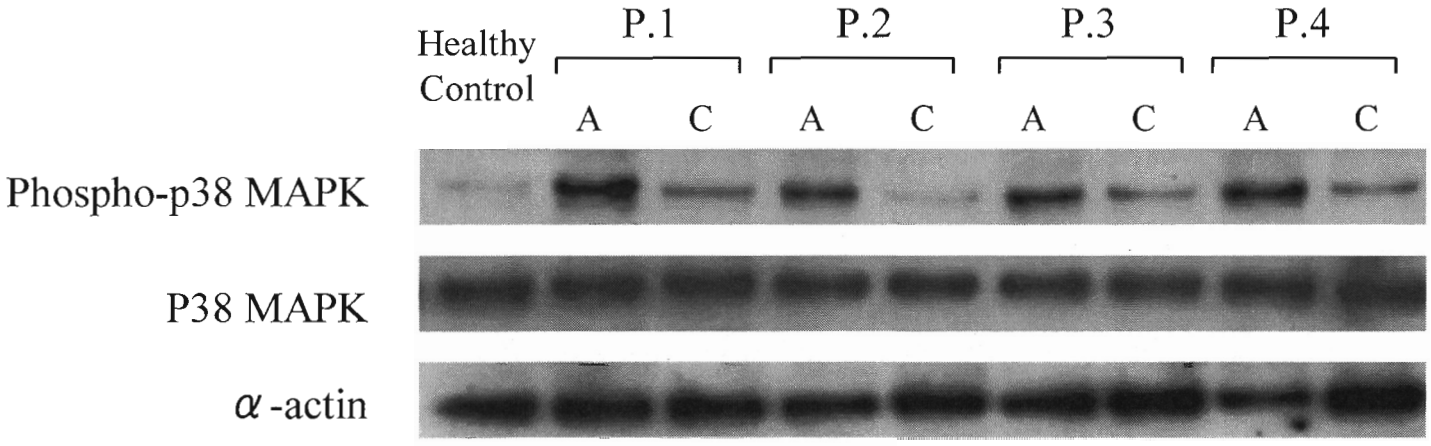
Healthy Control
P.1
P.2
P.3
P.4
A
C
A
C
A
C
A
C
Phospho-p38 MAPK
P38 MAPK
α-actin

Figure.1

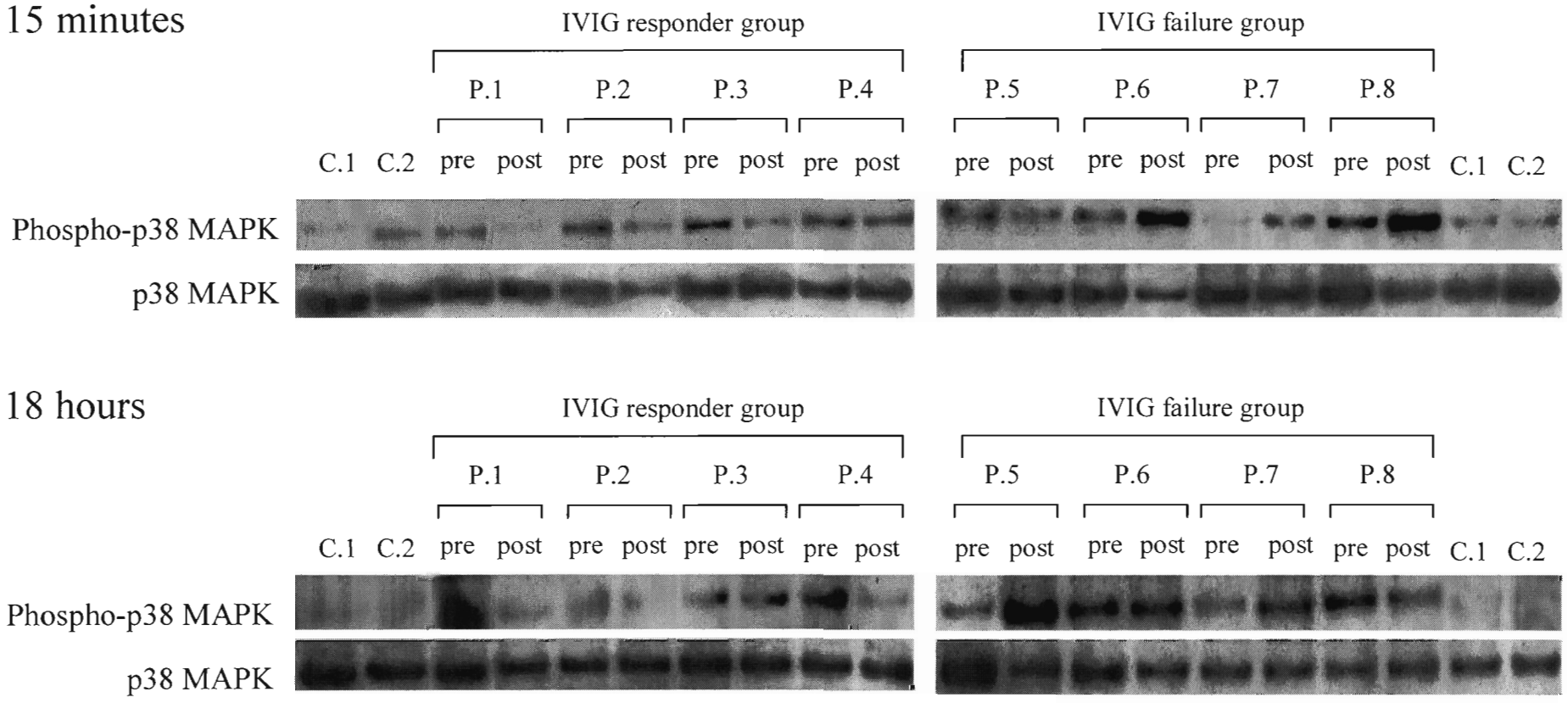
15 minutes
IVIG responder group
IVIG failure group
P.1
P.2
P.3
P.4
P.5
P.6
P.7
P.8
C.1
C.2
pre
post
Phospho-p38 MAPK
p38 MAPK
18 hours
IVIG responder group
IVIG failure group
P.1
P.2
P.3
P.4
P.5
P.6
P.7
P.8
C.1
C.2
pre
post
Phospho-p38 MAPK
p38 MAPK

Figure.2

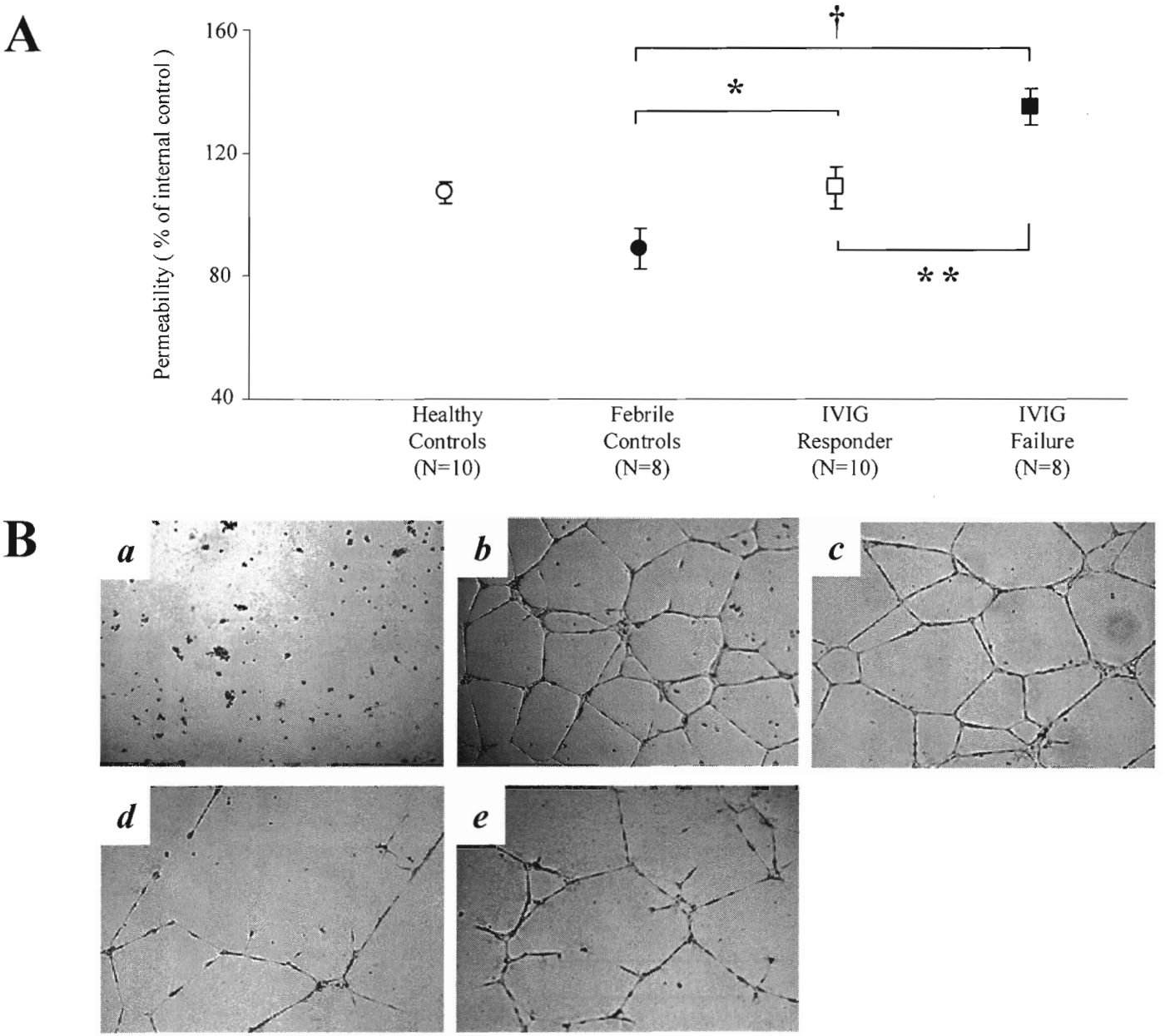
A
160
120
80
40
Permeability (% of internal control)
†
*
**
Healthy Controls (N=10)
Febrile Controls (N=8)
IVIG Responder (N=10)
IVIG Failure (N=8)
B
a
b
c
d
e

Figure.3

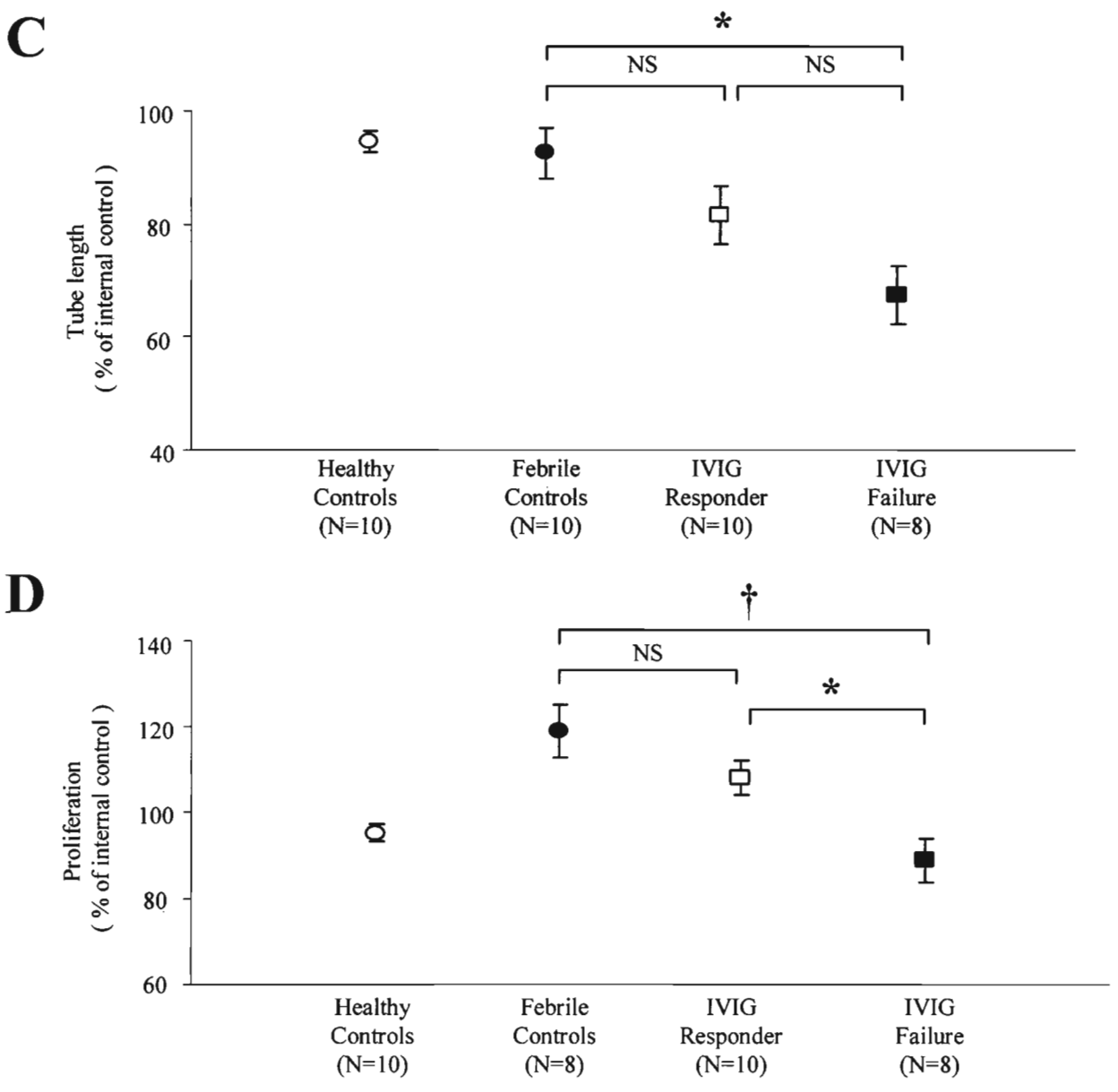
C
*
NS
NS
100
80
60
40
Tube length
(% of internal control)
Healthy Controls (N=10)
Febrile Controls (N=10)
IVIG Responder (N=10)
IVIG Failure (N=8)
D
†
NS
*
140
120
100
80
60
Proliferation
(% of internal control)
Healthy Controls (N=10)
Febrile Controls (N=8)
IVIG Responder (N=10)
IVIG Failure (N=8)

Figure.3

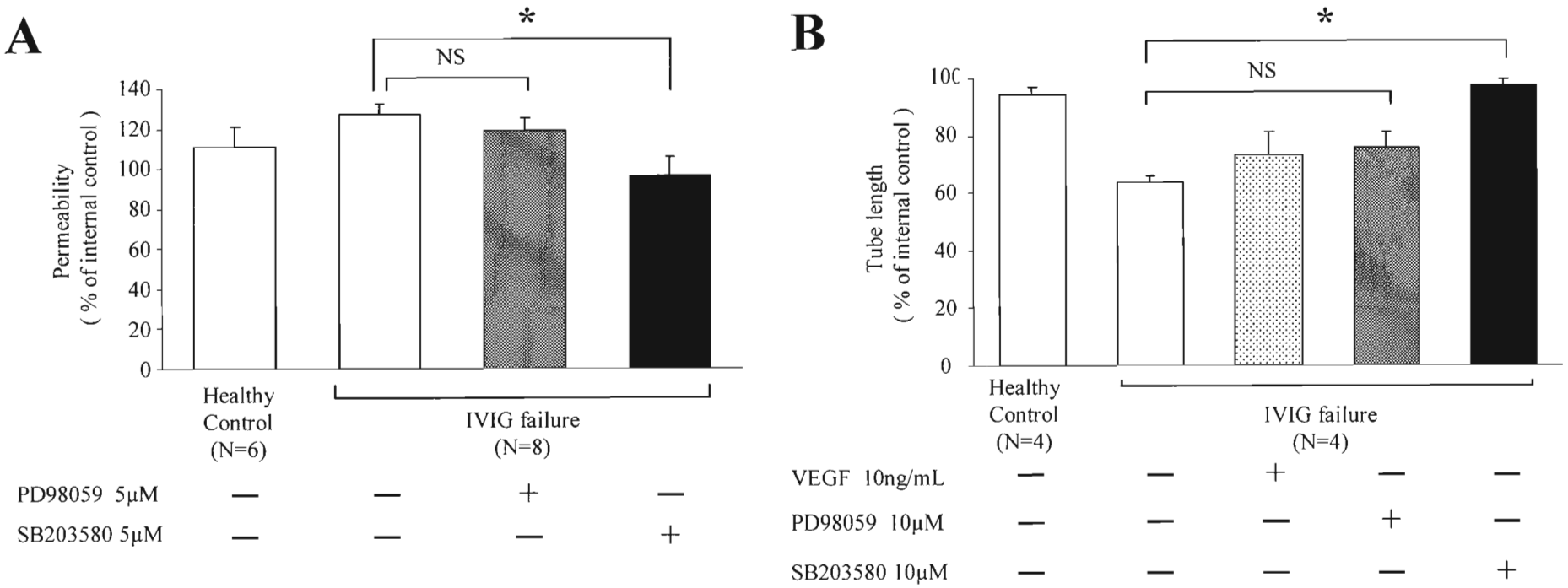
A
*
NS
Permeability
(% of internal control)
140
120
100
80
60
40
20
0
Healthy
Control
(N=6)
IVIG failure
(N=8)
PD98059 5μM
SB203580 5μM
B
*
NS
Tube length
(% of internal control)
100
80
60
40
20
0
Healthy
Control
(N=4)
IVIG failure
(N=4)
VEGF 10ng/mL
PD98059 10μM
SB203580 10μM
C
a
b
c
d
e

Figure.4

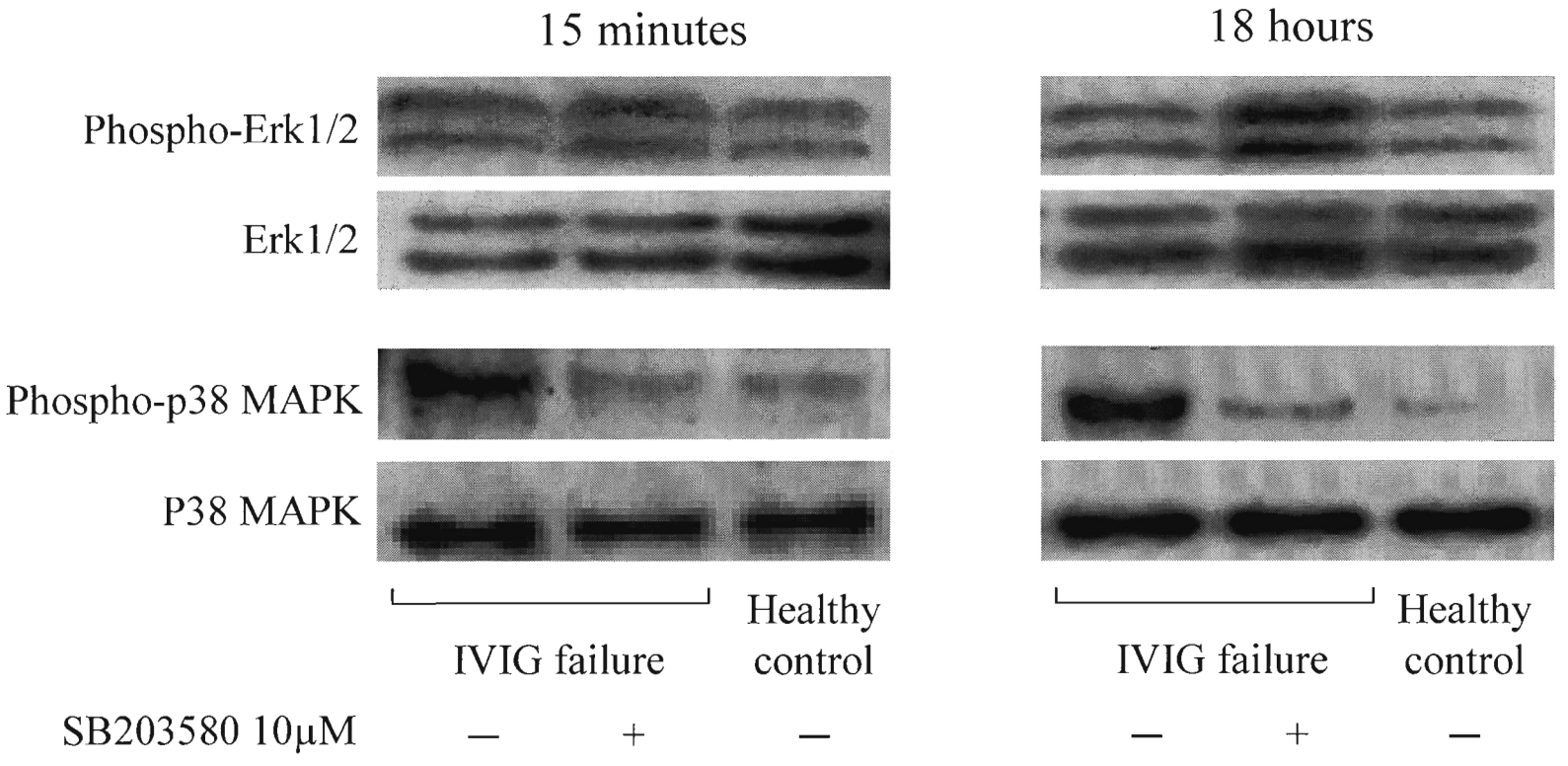
15 minutes
18 hours
Phospho-Erk1/2
Erk1/2
Phospho-p38 MAPK
P38 MAPK
IVIG failure
Healthy control
IVIG failure
Healthy control
SB203580 10μM
—
+
—
—
+
—

Figure.5