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C-reactive protein induces endothelial cell apoptosis and matrix metalloproteinase-9 production in human mononuclear cells: Implications for the destabilization of atherosclerotic plaque

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Abstract

C-reactive protein (CRP) has been suggested to directly induce the inflammatory response leading to the progression of atherosclerosis. However, recent *in vitro* studies raised the possibility that the effects of CRP are caused by biologically active contaminants such as sodium azide and endotoxin. In this study, we tested whether azide- and endotoxin-free CRP induces endothelial cell apoptosis and production of proinflammatory mediators. In human endothelial cells, CRP significantly inhibited cell proliferation and increased endothelial cell apoptosis evaluated by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling and caspase-3 activity assay, which is reversed by a function-blocking antibody to FcgammaRIIIB by 78%. Western blot analysis showed that CRP significantly attenuated flow-mediated activation of Akt, a key molecule for endothelial cell survival pathways. In human mononuclear cells, CRP-induced production of tumor necrosis factor-alpha (TNF-alpha), interleukin-1beta (IL-1beta), and matrix metalloproteinase-9 (MMP-9) in a concentration-dependent manner. This CRP-induced MMP-9 production was significantly inhibited by function-blocking antibodies to TNF-alpha, IL-1beta, and FcgammaRIIA. These findings suggest that CRP itself induces endothelial cell apoptosis and production of proinflammatory mediators. Because endothelial cell apoptosis and MMP-9 production are critical for the destabilization of atherosclerotic plaque, this study may provide insight into a role of CRP in the development of plaque rupture.

Keywords: C-reactive protein; Apoptosis; Matrix metalloproteinase; Endothelium; Leukocytes

1. Introduction

C-reactive protein (CRP) has been proposed to be an independent risk factor for atherosclerosis including ischemic heart disease, restenosis after percutaneous coronary intervention, stroke, and peripheral artery disease [1–6]. An immunohistological study showed that CRP is present in human coronary atheromatous lesions and its level positively correlates with the degree of progression of atherosclerosis [7]. Several studies have shown that CRP directly exhibits proinflammatory actions on vascular cells such as activation of the classical complement pathway, induction of adhesion molecules, and inhibition of nitric oxide production [8–10]. However, it remains controversial whether CRP is just a predictor of cardiovascular events or a direct mediator for atherosclerosis.

Recent works have suggested that antiproliferative, proapoptotic, and proinfalmmatory effects of commercial CRP on endothelial cells are caused by biologically active contaminants such as sodium azide and lipopolysaccharide (LPS), so called endotoxin [11,12]. On the other hand,

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conformational rearrangement in native CRP has been shown to promote proinflammatory actions on endothelial cells in azide- and endotoxin-free conditions [13]. In the preliminary study, we have found that azide- and endotoxin-free CRP promotes production of proinflammatory cytokines in human monocytic THP-1 cells. Moreover, receptors for CRP have been shown to be expressed in human endothelial cells and leukocytes [14,15]. Taken together, we hypothesized that CRP itself activates human endothelial cells and leukocytes in the absence of biologically active contaminants.

2. Materials and methods

2.1. Materials

Sodium azide-free recombinant human CRP (rhCRP) was obtained in a special order from Oriental Yeast. Endotoxin was removed from CRP solutions by using the Detoxigel column (Pierce Biochemicals) according to the manufacturer's instructions, and the endotoxin levels of all peptide solutions used in the experiments were below the detection limit (<0.001 ng/mL LPS). All other materials were from Sigma–Aldrich except where indicated.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion as previously described [16] and grown in medium MCDB131 supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin (P/S), and basic fibroblast growth factor (bFGF, Peprotech) in a humidified incubator. THP-1 cells (ATCC) were maintained in medium RPMI 1640 supplemented with 10% FCS and P/S. Prior to experiments, THP-1 cells were treated with 100 nM PMA for 12 h to induce the characteristics of macrophage. Peripheral blood mononuclear cells (PBMCs) were isolated by using density gradient centrifugation with SEPARATE-L (Muto-Kagakuyakuhin) according to manufacturer's instructions and resuspended in RPMI 1640 supplemented with 10% FCS and P/S. The percentage of monocytes in PBMCs was determined by flowcytometry (Becton Dickinson).

2.3. Cell proliferation of HUVECs

HUVECs (2×10^5 cells/dish) were seeded onto collagen-coated 3 cm dishes and cultured to subconfluency, and then the medium was exchanged for that without bFGF and cultured with or without CRP (10 μg/mL). After incubation for the indicated times, cells were detached and cell numbers were counted using a Coulter Counter (Beckman Coulter).

2.4. Determination of apoptosis in HUVECs

HUVECs (1×10^4 cells/well) were seeded onto collagen-coated 8-well chamber slides (Nunc) and cultured to subconfluency. Then cells were incubated with or without CRP (10 μg/mL) for 24 h. Apoptosis of HUVECs was evaluated by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) using *in situ* Apoptosis Detection Kit (Takara Bio). Briefly, after treatment with or without CRP, HUVECs were washed in PBS and fixed with 4% paraformaldehyde, then incubated in permeabilization buffer and FITC-labeling safe buffer with deoxynucleotidyltransferase. After washing in PBS, cells were subjected to fluorescent microscopic examination. TUNEL positive cells were microscopically counted and the percentage of TUNEL positive cells was calculated. For quantification of caspase-3 activity, capase-3 activity assay was performed with the use of CPP32/Caspase-3 Colorimetric Protease Assay Kit (Medical & Biological Laboratories) according to the manufacturer's instructions. In some experiments, HUVECs were incubated with a function-blocking antibody to FcgammaRIIA or FcgammaRIIIB (R&D Systems) in addition to CRP. After treatment, HUVECs were washed in ice-cold PBS, and cell lysates were prepared by flash-freezing and thawing in lysis buffer. Cell lysates were incubated in reaction buffer with DEVD, a caspase recognizing sequence, conjugated chromophore *p*-nitroanilide (*p*NA) substrate, and then subjected to the quantification of *p*NA light emission using a microtiter plate reader at 405 nm. Comparison of the absorbance of *p*NA from an apoptotic sample with an uninduced control allowed determination of the fold increase in caspase-3 activity. Background reading from cell lysates and buffers was subtracted from the readings of both induced and uninduced samples before calculating fold increase in caspase-3 activity.

2.5. Western blot analysis

HUVECs were incubated with or without CRP (10 μg/mL) for 12 h, and then exposed to flow (shear stress of 12 dyn/cm^2) for 5 min using a cone-plate apparatus as previously described [17]. After exposure to flow, cell lysates were prepared, subjected to SDS-PAGE, and transferred to nitrocellulose membranes (Amersham Biosciences). The membrane was blocked for 1 h at room temperature with PBS containing 2% BSA and 0.05% Tween 20. The blots were incubated overnight at 4 °C or for 4 h at room temperature with an anti-phospho-Akt antibody (Santa Cruz Biotechnology), followed by incubation for 1 h with a secondary, horseradish peroxidase-conjugated antibody, and then reprobed with an anti-Akt antibody. Immunoreactive bands were visualized using ECL (Amersham Biosciences). Densitometry analysis of immunoreactive bands was performed using NIH Image 1.62. All experiments were performed at least three times.

2.6. Measurement of production of tumor necrosis factor-alpha (TNF-alpha), interleukin-1beta (IL-1beta), and matrix metalloproteinase-9 (MMP-9) in human mononuclear cells

After incubation with or without CRP (5 μg/mL) for 12 h, supernatants were withdrawn from the culture dishes and stored at 4 °C. Concentrations of TNF-alpha, IL-1beta, and MMP-9 in the culture supernatants were measured by ELISA using the quantitative sandwich enzyme immunoassay technique for TNF-alpha and IL-1beta (R&D Systems), and by using the Biotrak™ MMP-9 assay (Amersham Biosciences) according to the manufacturer's instructions. In some experiments, human mononuclear cells were incubated with a function-blocking antibody to TNF-alpha, IL-1beta, or FcgammaRIIA (R&D Systems). The intra- and inter-assay coefficients of variation were 5.0% and 7.3% for TNF-alpha assay, 3.4% and 4.1% for IL-1beta assay, and 4.9% and 8.1% for MMP-9 assay, respectively, according to the manufacturer's instructions.

2.7. Statistical analysis

Data are shown as mean ± S.D. Differences were analyzed with paired *t*-tests between two groups, and one-way analysis of variance (ANOVA) among more than three groups. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Inhibition of endothelial cell proliferation by CRP

Proliferation of HUVECs incubated with CRP was significantly inhibited compared to that of control cells cultured with vehicle (2.2 ± 0.08-fold versus 2.7 ± 0.13-fold at 24 h; 2.3 ± 0.09-fold versus 3.4 ± 0.15-fold at 48 h, respectively; $p < 0.01$; Fig. 1).

3.2. Induction of endothelial cell apoptosis and attenuation of Akt activation by CRP

To examine the involvement of endothelial cell apoptosis in CRP-induced inhibition of cell proliferation, we performed TUNEL and caspase-3 activity assay. Percentage of TUNEL positive cells significantly increased in HUVECs incubated with CRP compared to control cells (Table 1). CRP induced increase of caspase-3 activity was quantitatively confirmed

Table 1
CRP-induced increase in TUNEL positive cells and caspase-3 activity

	Control	CRP	*p*-Value
TUNEL positive cells (%)	0.47 ± 0.10	0.77 ± 0.10	<0.01
Caspase-3 activity (fold increase)	1.0 ± 0	1.16 ± 0.07	<0.05

Data are mean ± S.D., $n = 3$–4.

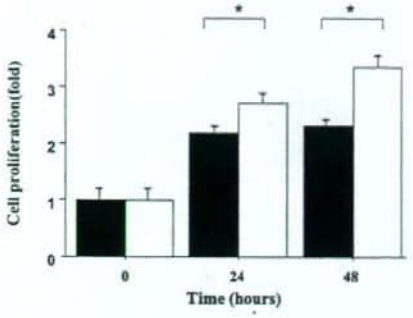

Fig. 1. Time-dependent effects of CRP on HUVEC proliferation. HUVECs (2×10^5 cells/dish) were seeded onto collagen-coated 3 cm culture dishes and cultured to subconfluency for 12–24 h. Then the medium was changed for that without bFGF and cells were cultured with (■) or without (□) CRP (10 μg/mL) for 0, 24, and 48 h. Cells were detached after incubation for the indicated times and cell numbers were counted using a Coulter Counter. HUVEC proliferation was assessed as fold increase to the initial cell number (2×10^5 cells). Results are mean ± S.D., $n = 3$; $^*p < 0.01$.

by caspase-3 activity assay (Table 1). To clarify the mechanism by which endothelial cell apoptosis is induced, we investigated the effect of CRP on Akt activation. Akt has been shown to play a critical role in endothelial cell survival pathways and to be activated by flow [18]. Akt was markedly phosphorylated by flow in HUVECs incubated with vehicle, whereas this flow-induced phosphorylation of Akt was significantly attenuated in HUVECs incubated with CRP as evaluated by densitometry analysis ($p < 0.01$; Fig. 2).

3.3. Production of proinflammatory cytokines and MMP-9 by CRP in human mononuclear cells

To address a role of CRP in the inflammatory response, we examined the effect of CRP on production of proinflammatory cytokines and MMP-9. MMP-9 has been shown to be a key mediator for the destabilization of atherosclerotic plaque [19]. In PBMCs, CRP significantly increased produc-

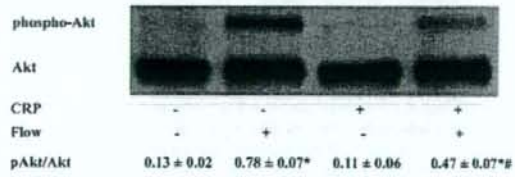

Fig. 2. Effects of CRP on phosphorylation of Akt by flow in HUVECs. After incubation with or without CRP (10 μg/mL) for 12 h, HUVECs were exposed to flow (shear stress of 12 dyn/cm^2) for 5 min and cell lysates were prepared as described in Section 2. Activation of Akt was assessed by Western blot using an anti-phospho-Akt antibody, and then blots were reprobed with an anti-Akt antibody to show equal protein loading. Immunoreactive bands were analyzed by NIH Image 1.62 and ratios of densitometry analysis (phospho-Akt/Akt) were calculated. Results shown are representative of three separate experiments. Data of pAkt/Akt are mean ± S.D., $n = 3$; $^*p < 0.01$ vs. static, $^\#p < 0.01$ vs. under flow without CRP.

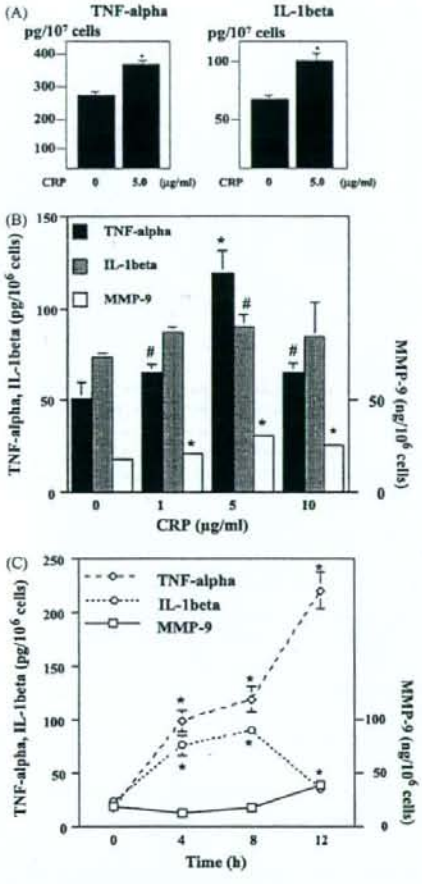

Fig. 3. (A) Effect of CRP on production of proinflammatory cytokine in PBMCs. PBMCs were seeded onto 6-well plates (1×10^6 cells/well) and cultured in RPMI 1640 supplemented with 10% FCS for 12 h. Then cells were incubated in serum free medium with or without CRP (5 μg/mL). Control cells were incubated with vehicle only. After 12 h incubation, samples were collected and concentrations of TNF-alpha and IL-1beta in the culture supernatants were measured by ELISA. Results are mean ± S.D., $n = 3$; $^*p < 0.01$. (B) Dose response of CRP-induced production of proinflammatory cytokines and MMP-9 in THP-1 cells. THP-1 cells were seeded onto 6-well plates (1×10^6 cells/well) and cultured as in (A). Then cells were incubated in serum free medium with the indicated concentrations of CRP. Control cells were incubated with vehicle only. After 12 h incubation, samples were collected and concentrations of TNF-alpha, IL-1beta, and MMP-9 in the culture supernatants were measured by ELISA and the Biotrak™ MMP-9 assay. Results are mean ± S.D., $n = 4$; $^*p < 0.01$, $^\#p < 0.05$ vs. control. The S.D. bars for MMP-9 are not drawn because of small S.D. values (ranged 0.53–1.0 ng/10^6 cells). (C) Time course of CRP-induced production of proinflammatory cytokines and MMP-9 in THP-1 cells. THP-1 cells were seeded and cultured as in (A), and then incubated in serum free medium with CRP (5 μg/mL) for the indicated times. Concentrations of TNF-alpha, IL-1beta, and MMP-9 in the culture supernatants were measured as in (B). Results are mean ± S.D., $n = 4$; $^*p < 0.01$ vs. control. The S.D. bars for MMP-9 are not drawn because of small S.D. values (ranged 0.56–1.81 ng/10^6 cells).

tion of TNF-alpha (365 ± 18 pg/10^7 cells with CRP versus 277 ± 21 pg/10^7 cells with vehicle; $p < 0.01$) and IL-1beta (99 ± 7 pg/10^7 cells with CRP versus 71 ± 5 pg/10^7 cells with vehicle; $p < 0.01$) (Fig. 3A). CRP-induced production of TNF-alpha and IL-1beta was also confirmed in THP-1 cells. CRP stimulated production of TNF-alpha and IL-1beta in a concentration-dependent manner with peak at 5 μg/mL (Fig. 3B). Because the percentage of monocytes in PBMCs was found to be 10% by flowcytometry, cell number of monocytes in PBMCs (10^7 cells/well) corresponded to that of THP-1 cells used in the experiments. Next, we investigated whether CRP-induced production of TNF-alpha and IL-1beta could contribute to the production of MMP-9. CRP induced a significant increase of MMP-9 production (9.2 ± 1.3 ng/10^7 cells with CRP versus 6.6 ± 0.9 ng/10^7 cells with vehicle; $p < 0.01$; Fig. 4A). This CRP-induced production of MMP-9 was concentration-dependent (Fig. 3B), and production of TNF-alpha and IL-1beta preceded that of MMP-9 (Fig. 3C). Furthermore, a function-blocking antibody for TNF-alpha almost completely blocked CRP-induced MMP-9 production, and an anti-IL-1beta antibody inhibited its production by 75% (Fig. 4A).

3.4. *Involvement of CRP receptors, FcgammaRIIA and FcgammaRIIIB in MMP-9 production and endothelial cell apoptosis induced by CRP*

To clarify whether the actions of CRP were receptor-mediated or not, we used function-blocking antibodies to FcgammaRIIA (anti-FcgammaRIIA) and FcgammaRIIIB (anti-FcgammaRIIIB). Because THP-1 cells do not express FcgammaRIII [14], we examined the effect of anti-FcgammaRIIA on MMP-9 production in THP-1 cells. As shown in Fig. 4B, anti-FcgammaRIIA significantly inhibited CRP-induced MMP-9 production by 72%. Next, we investigated the effects of anti-FcgammaRIIA and anti-FcgammaRIIIB on HUVEC apoptosis. Anti-FcgammaRIIIB, but not anti-FcgammaRIIA, significantly inhibited CRP-induced HUVEC apoptosis by 78% as assessed by caspase-3 activity assay (Fig. 4C).

4. Discussion

The present study shows that azide- and endotoxin-free CRP induces endothelial cell apoptosis via FcgammaRIIIB and promotes production of TNF-alpha, IL-1beta, and MMP-9 through FcgammaRIIA in human mononuclear cells. CRP-induced endothelial cell apoptosis may be mediated by attenuation of Akt activation, and CRP-induced production of TNF-alpha and IL-1beta are required for subsequent production of MMP-9. These findings suggest that CRP directly induces endothelial cell apoptosis and production of proinflammatory mediators in human mononuclear cells.

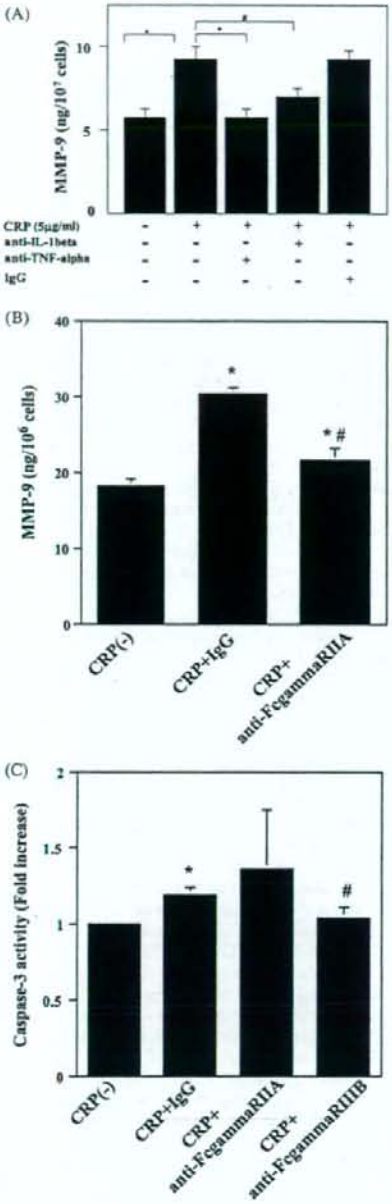

Fig. 4. (A) Effect of CRP on production of MMP-9 in PBMCs and its inhibition by function-blocking antibodies to TNF-alpha and IL-1beta. PBMCs were treated as in Fig. 3A and incubated with vehicle only, CRP (5 μg/mL) and IgG, CRP and anti-TNF-alpha, or CRP and anti-IL-1beta for 12 h. Then samples were collected and concentrations of MMP-9 in the culture supernatants were determined by using the Biotrak™ MMP-9 assay. Results are mean ± S.D., $n=3$; $^{*}p<0.01$, $^{\#}p<0.05$. (B) Effect of anti-FcgammaRIIA on CRP-induced MMP-9 production in THP-1 cells. THP-1 cells were treated as in Fig. 3A and incubated with vehicle only, CRP (5 μg/mL) and control IgG, or CRP and anti-FcgammaRIIA. Then samples were collected and concentrations of MMP-9 in the culture supernatants were determined as in (A). Results are mean ± S.D., $n=4$; $^{*}p<0.01$ vs. control, $^{\#}p<0.01$ vs. CRP + IgG. (C) Effects of anti-FcgammaRIIA and anti-FcgammaRIIIB on CRP-induced HUVEC apoptosis. HUVECs were cultured in 60 mm culture dishes and incubated with vehicle only, CRP (10 μg/mL) and control IgG, CRP and anti-FcgammaRIIA, or CRP and anti-FcgammaRIIIB for 24 h. Then cell lysates were prepared and caspase-3 activity assay was performed as described in Section 2. Results are mean ± S.D., $n=4$; $^{*}p<0.01$ vs. control, $^{\#}p<0.05$ vs. CRP + IgG.

Because endothelial cell apoptosis and increased production of MMP-9 contribute to the destabilization of atherosclerotic plaque [20,21], this study may provide insight into a role of CRP in the development and progression of plaque rupture.

There is growing evidence that CRP is not only an independent predictor of cardiovascular events but also a direct mediator for atherosclerosis [1–10]. However, recent works addressed criticism that the *in vitro* effects of CRP on vascular cells may be due to biologically active contaminants such as sodium azide or endotoxin [11,12]. Taylor et al. reported that azide and LPS, but never CRP itself, were responsible for endothelial cell activation events [11]. Liu et al. also showed that commercial CRP and sodium azide, but not dialyzed commercial CRP to remove azide, exhibited proapoptotic effects on various human endothelial cell types [12]. On the other hand, Khreiss et al. demonstrated that loss of the cyclic pentameric structure of CRP, resulting in formation of monomeric CRP (mCRP), was required for proinflammatory actions on human endothelial cells in azide- and endotoxin-free conditions [13]. In the present study, we have found that CRP itself activates HUVECs and human mononuclear cells using azide- and endotoxin-free rhCRP, which is indistinguishable from native CRP [22], as described in Section 2. Our results have clearly demonstrated the direct proinflammatory effects of CRP on human mononuclear cells in addition to its proinflammatory actions on endothelial cells shown by Khreiss et al. [13]. Consistent with our data, Verma et al. showed that rhCRP induces apoptosis in human endothelial cells and in endothelial progenitor cells [10,23]. However, it is unclear whether rhCRP itself induces these biological actions because native or rhCRP dissociates into mCRP within a few hours after binding to plasma membrane [13]. Furthermore, mCRP, but not native CRP itself, has been shown to play an important role in proinflammatory actions on human endothelial cells and in delayed apoptosis of human neutrophils [13,24]. Considering these findings and our experimental conditions (12–24 h incubation with rhCRP), CRP-induced apoptosis of HUVECs and proinflammatory effects of CRP on human mononuclear cells observed in this study may be induced by mCRP rather than rhCRP itself.

It remains to be elucidated whether CRP would directly affect Akt phosphorylation or not. We previously showed that PI3-kinase is an upstream mediator of shear stress-induced Akt activation in endothelial cells [18]. Liu et al. demon-

strated that the SH-2-containing inositol 5-phosphatase negatively regulates growth factor receptor-mediated Akt activation by counteracting the action of PI3-kinase [25]. Furthermore, Mineo et al. reported that FcgammaRIIB mediates CRP inhibition of endothelial nitric oxide synthase (eNOS) via protein phosphatase 2A (PP2A) [26]. Taken together, it is likely that negative modulation of PI3-kinase by phosphatases is involved in CRP-induced attenuation of Akt phosphorylation.

In the present study, the anti-TNF-alpha antibody and the anti-IL-1beta antibody blocked CRP-induced MMP-9 production by nearly 100% and by 75%, respectively. Philip and Epstein demonstrated that TNF-alpha induces IL-1beta production in human monocytes [27]. We also confirmed that the anti-TNF-alpha antibody blocked CRP-induced IL-1beta production by 60% in THP-1 cells (data not shown). Moreover, Zhang et al. reported that both TNF-alpha and IL-1beta induce monocyte production of MMP-9 [28], which is consistent with our data. Based on these findings, TNF-alpha may mediate CRP-induced IL-1beta production and play a central role in MMP-9 production induced by CRP. However, why CRP-induced cytokine production peaked at a lower level of CRP concentration is unclear, but could involve down-regulation or attenuation of its production by anti-inflammatory cytokine IL-10 at a higher level [29].

It has been shown that FcgammaRI and FcgammaRII, which bind to CRP, are expressed in human endothelial cells and leukocytes [14,15], and that FcgammaRIII, which is a receptor for mCRP, is also expressed in human endothelial cells [13]. In this study, we have clearly demonstrated that FcgammaRIIA mediates CRP-induced production of MMP-9 in human mononuclear cells and that FcgammaRIIIB plays a critical role in CRP-induced apoptosis in human endothelial cells. Interestingly, it is likely that CRP suppression of eNOS activity and induction of apoptosis in human endothelial cells are mediated via different receptors, FcgammaRIIB and FcgammaRIIIB. FcgammaRIIB has been reported to be involved in dephosphorylation of eNOS at Ser1179 by PP2A [26], and FcgammaRIIIB mediates the increase of caspase-3 activity presumably through attenuation of Akt activation.

A recent study from Pepys et al. has demonstrated that administration of a specific small-molecule inhibitor of CRP, 1,6-bis(phosphocholine)-hexane, to rats undergoing acute myocardial infarction abrogates the increase in infarct size and cardiac dysfunction produced by injection of human CRP [30]. In the present study, CRP directly induces endothelial apoptosis presumably due to attenuation of Akt activation, and mediates production of MMP-9 through induction of TNF-alpha and IL-1beta *in vitro*, suggesting an active role of CRP in the destabilization of atherosclerotic plaque leading to the development and progression of plaque rupture. Thus, CRP itself can be a direct mediator for the proinflammatory response and prevention of CRP production may be a promising therapeutic approach for cardiovascular diseases.

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Relationship among VEGF, VEGF receptor, AGEs, and macrophages in proliferative diabetic retinopathy

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ABSTRACT

Purpose: We studied the roles of vascular endothelial growth factor (VEGF), its receptor (flt-1), advanced glycation end products (AGEs), and macrophages in the development of proliferative diabetic retinopathy.

Methods: Ocular fluid and small specimens of iris and neovascular membrane were obtained from 30 patients who underwent vitreous surgery (19 eyes with proliferative diabetic retinopathy [PDR], 11 eyes with non-diabetic ocular diseases). VEGF and AGE levels in ocular fluid were assayed by ELISA. Immunohistochemical studies of VEGF, flt-1, AGEs, and macrophage were performed on the ocular tissues.

Results: The mean VEGF and AGE levels in the vitreous (695.7 pg/ml and 2.4 mg/ml, respectively) were significantly higher in diabetic than in non-diabetic eyes (25.9 pg/ml, $p = 0.0007$ and 1.3 mg/ml, $p = 0.005$, respectively). Likewise, in the aqueous humor, VEGF and AGE levels were significantly higher in diabetic than in non-diabetic eyes. VEGF levels in the vitreous and aqueous humor were correlated significantly ($r = 0.6$; $p = 0.02$), but AGEs were not. The VEGF levels were not correlated with AGE levels in the aqueous or vitreous. In the iris, VEGF, AGEs, and macrophages were stained more prominently in the specimens from patients with diabetes than from patients without diabetes, while flt-1 staining did not differ. The Neovascular membranes were stained much more prominently for all (VEGF, flt-1, AGEs and macrophages) even when compared with the iris from patients with diabetes.

Conclusions: By analyzing aqueous and vitreous humor, proliferative membranes, and iris from the same patients, the current clinical study strongly supports previous reports that showed the role of VEGF, macrophages, and AGEs in the development of diabetic proliferative retinopathy. From the results of the current study, we showed that flt-1 plays an important role in the development of retinal neovascular membranes but the role is uncertain in the iris and retina.

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1. Introduction

The direct cause of visual loss in diabetic retinopathy, the disastrous end result of this complication, is retinal neovascularization. In its early stage, retinal neovascularization may be treated with panretinal photocoagulation and even when advanced the neovascular membrane can be removed by vitreous surgery. However, neovascular glaucoma, secondary glaucoma due to iris neovascularization, is a complication for which no treatment is effective in most cases. Recent studies have hypothesized that vascular endothelial growth factor (VEGF) and accumulation of advanced glycation end products (AGEs) play an important role in the initiation and development of retinal neovascularization as well as the iris [1–8]. VEGF was elevated not only in the late stage [1,8] but also in the early stage of diabetic retinopathy [9]. VEGF-induced leukocyte adhesion to the retinal vessels was shown to result in blood–retinal barrier breakdown [10]. VEGF was initially shown to be produced by retinal pigment epithelial cells; however, more recently the role of VEGF-producing monocyte/macrophages that infiltrate into the vitreous is considered to be even more important in the subsequent migration of retinal vascular endothelial cells [11–17]. In the present report, we studied VEGF, flt-1 (a VEGF receptor), AGEs, and macrophage simultaneously to clarify their inter-relationship in eyes with proliferative diabetic retinopathy (PDR).

2. Materials and methods

Ocular fluid (aqueous humor and vitreous humor), a small iris specimen, and neovascular membrane were obtained from 30 patients undergoing vitreous surgery (19 with PDR, six with a macular break, four with an idiopathic preretinal membrane, and one with a rhegmatogenous retinal detachment). The mean age of the patients with diabetes was 55.9 years (range, 28–78) and that of the patients without diabetes was 66.9 years (range, 59–84). The tenets of the Declaration of Helsinki were followed. Institutional review board approval was obtained and written informed consent was obtained from all patients for the collection of ocular fluid, iris specimen, and neovascular membrane.

Aqueous humor was obtained through a 30-G needle attached to a 5-ml disposable syringe. The iris specimen was obtained by peripheral iridectomy to avoid a postoperative pupillary block. The vitreous humor was obtained under air infusion without contamination by artificial intraocular fluid (BSS plus, Alcon, Fort Worth, TX, USA). VEGF levels were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit (human VEGF EIA Kit, Immuno-Biological Laboratories Co. Ltd., Fujioka, Japan) (PDR $n = 19$; controls $n = 11$). AGE (N^{ε}-(carboxymethyl) lysine, CML) levels in the vitreous were measured by ELISA using monoclonal antibody against CML designated as 6D12 (PDR $n = 13$; control $n = 8$). CML was not assayed in all samples due to the limited volume of the vitreous and aqueous samples available.

The iris specimens and neovascular membranes were fixed in 15% formalin, embedded in paraffin, sectioned to 4 μm and stained with hematoxylin and eosin for conventional histopathologic examination or immunostained for flt-1 (a polyclonal antibody specific for flt-1, 1:1000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), VEGF (a monoclonal antibody for human VEGF (147), 1:1000 dilution, Santa Cruz Biotechnology, Inc.), CML (a monoclonal antibody 6D12 [18], 1:3000 dilution), and macrophage (monoclonal antibody against human macrophage, 1:20 dilution, HAM56, DakoCytomation, Carpinteria, CA, USA). The neovascular membrane was removed using a high speed cutter. It was often difficult to obtain en-bloc sampling of the neovascular membrane during the surgery so the number of samples obtained was limited. The immunohistochemical procedures were based on the standard avidin–biotin horseradish peroxidase method using each antibody and developed with AEC Substrate Chromogen (DakoCytomation, Carpinteria, CA, USA). Bovine serum was used as a primary antibody for negative control of the immunostaining.

3. Results

The mean VEGF level in the vitreous humor was higher in PDR eyes ($n = 19$, 695.7 pg/ml) than in non-diabetic eyes ($n = 9$, 25.9 pg/ml) ($p = 0.0007$ by Mann–Whitney U-test) (Fig. 1). The mean VEGF level in the aqueous humor was also higher in PDR eyes ($n = 16$, 397.4 pg/ml) than in non-diabetic eyes ($n = 7$, 46.9 pg/ml) ($p = 0.003$ by Mann–Whitney U-test) (Fig. 2). VEGF levels in the aqueous humor were significantly correlated with those in the vitreous humor ($n = 15$, $r = 0.6$; $p = 0.02$ by Pearson's correlation coefficient test) (Fig. 3). The mean AGE levels in both the vitreous humor and aqueous humor were higher in PDR eyes ($n = 13$, 2.4 mg/ml in vitreous; $n = 13$, 1.5 mg/ml in aqueous) than in non-diabetic eyes ($n = 6$, 1.3 mg/ml in vitreous; $n = 4$, 0.93 mg/ml in aqueous) ($p = 0.005$ and 0.04, respectively, by Student's t-test) (Figs. 4 and 5). However, AGE levels in the aqueous humor were not correlated with those in the vitreous humor ($n = 10$, $r = 0.02$; $p = 0.95$ by Pearson's correlation coefficient test) (Fig. 6). AGE levels also were not correlated with VEGF levels in both the aqueous humor ($n = 13$, $r = 0.18$; $p = 0.52$ by Spearman's correlation

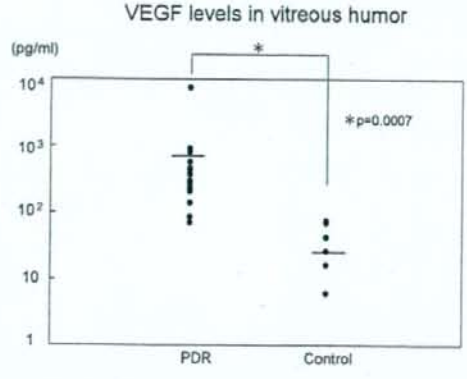

Fig. 1 – VEGF levels in the vitreous humor in PDR ($n = 19$) and non-diabetic ($n = 9$) eyes undergoing vitreous surgery. The VEGF levels are significantly higher in PDR than in non-diabetic eyes. *$p = 0.0007$ by the Mann–Whitney U-test.

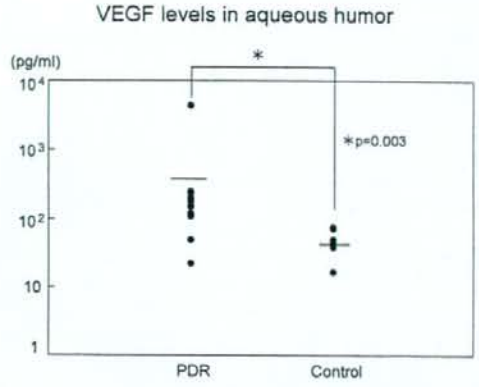

Fig. 2 – VEGF levels in the aqueous humor in PDR ($n = 16$) and non-diabetic ($n = 7$) eyes undergoing vitreous surgery. The VEGF levels are significantly higher in PDR than in non-diabetic eyes. *$p = 0.003$ by the Mann–Whitney U-test.

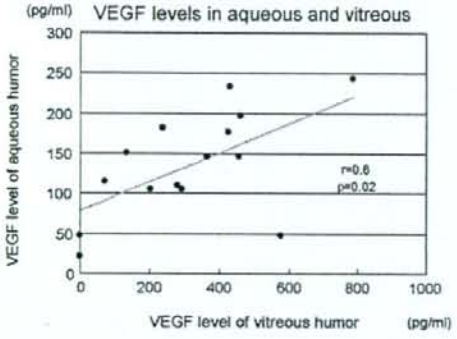

Fig. 3 – Relationship between the aqueous and vitreous VEGF levels in PDR ($n = 15$). $r = 0.6$; $p = 0.02$ by Pearson's correlation coefficient test.

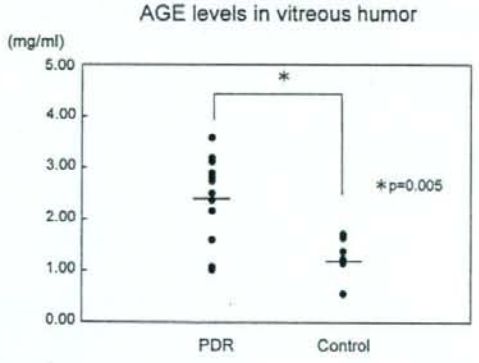

Fig. 4 – AGE levels in the vitreous humor in PDR ($n = 13$) and non-diabetic ($n = 6$) eyes undergoing vitreous surgery. The AGE levels are significantly higher in PDR than in non-diabetic eyes. *$p = 0.005$ by Student's t-test.

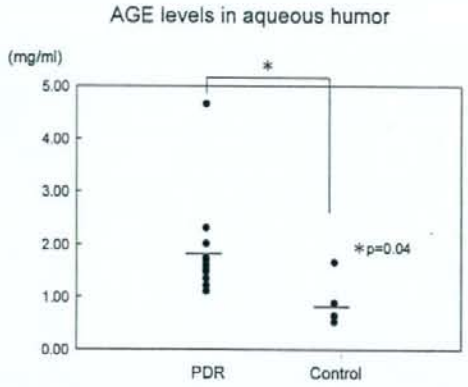

Fig. 5 – The AGE levels in the aqueous humor in PDR ($n = 13$) and non-diabetic ($n = 4$) eyes undergoing vitreous surgery. The AGE levels are significantly higher in PDR than in non-diabetic eyes. *$p = 0.04$ by Student's t-test.

coefficient test) (Fig. 7) and the vitreous humor ($n = 13$, $r = 0.33$; $p = 0.26$ by Spearman's correlation coefficient test) (Fig. 8).

Flt-1 staining of the iris varied independent of the presence or absence of diabetes. Among the 19 diabetic eyes, 2 (10.5%) were negative for staining and 17 (89.5%) were positive, while among 10 non-diabetic samples, 4 (40.0%) were negative and 6 (60.0%) were positive (Fig. 9). There was no significant difference in flt-1 staining between the diabetic and non-diabetic groups ($p = 0.09$).

VEGF staining was observed in the vascular endothelium of all diabetic irises in the area where adjacent macrophages also were stained (Fig. 10). AGE staining of extracellular substances was also marked in the diabetic irises (Fig. 10). In contrast, immunostaining of VEGF, AGEs, and macrophage was less prominent in the non-diabetic irises (Fig. 11). In neovascular membranes, extensive flt-1 staining at the vascular endothelium was observed in all cases ($n = 3$) (Fig. 12). We also observed in the neovascular membranes extensive VEGF staining at the vascular endothelium and macrophages and AGE accumulation in the extracellular substances (Fig. 13).

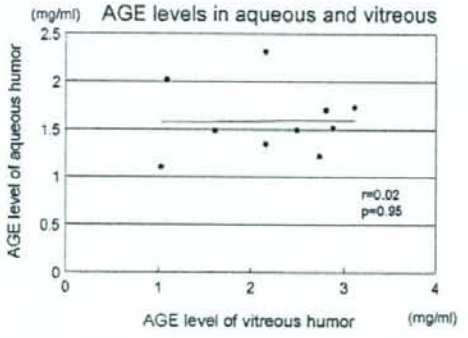

Fig. 6 – Relationship between aqueous and vitreous AGE levels in PDR ($n = 10$). $r = 0.2$; $p = 0.95$ by Pearson's correlation coefficient test.

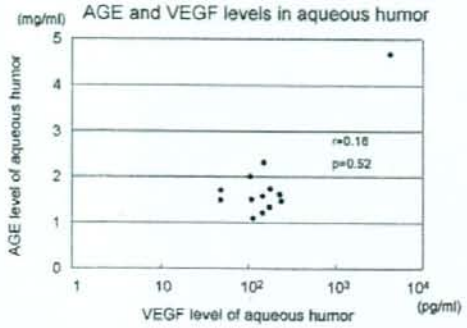

Fig. 7 – Relationship between aqueous AGE and VEGF levels in PDR ($n = 13$). $r = 0.18$; $p = 0.52$ by Spearman's correlation coefficient test.

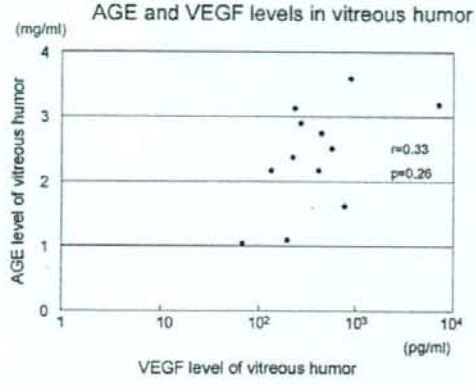

Fig. 8 – Relationship between vitreous AGE and VEGF levels in PDR ($n = 13$). $r = 0.33$; $p = 0.26$ by Spearman's correlation coefficient test.

4. Discussion

Retinopathy and rubeosis are two serious ocular complications in diabetes that ultimately may result in blindness. VEGF has been established as one of the most important factors that initiates those ocular complications. Major sources of VEGF in the retina were initially considered to be ischemic retina, including retinal pigment epithelial cells [19] and more recently infiltrating macrophages [20]. The role of AGEs as a stimulant that induces the production and secretion of VEGF by these cells also has been well documented [21]. Several studies have reported higher levels of VEGF and AGEs in diabetic compared to non-diabetic eyes and this was also true in the current study [22,23]. In addition, however, we studied the distribution of VEGF, flt-1 (a receptor for VEGF), and AGEs within the same eyes, which had not been done previously.

VEGF levels in the aqueous humor and vitreous humor were well correlated and were generally higher in the latter, which agrees well with the previous report that the major production site of VEGF is the retina [8,24].

Expression of flt-1 in the iris varied both in diabetic and non-diabetic eyes, while it was very prominent in all of the neovascular membranes from diabetic eyes with PDR. There have been several reports using animal models related to flt-1 expression, the results of which are somewhat inconsistent with our current observation. A murine model of ischemia-induced retinal neovascularization showed that KDR/flk-1 (another VEGF receptor) expression increased, but flt-1 expression was almost the same as in control animals [25]. In a rat model, the VEGF/VEGF receptor system, including both KDR/flk-1 and flt-1, was not up-regulated in early diabetic retinopathy [26]. Those studies suggested that flt-1 does not seem to participate in the retinal neovascularization in these animal models of early diabetic retinopathy or ischemic retinopathy. Because we obtained tissue specimens only at surgery, we did not have retinal samples. Accordingly, the expression of flt1 in the retina itself was not examined. Thus, we cannot be certain about regulation of flt-1 in the retina. It would seem that it is not up-regulated, since flt-1 was not increased in diabetic irises.

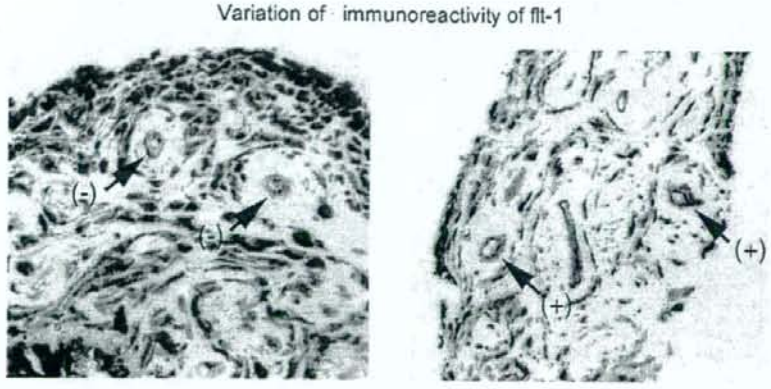

Fig. 9 – Variation of immunoreactivity of flt-1 in diabetic iris. The photograph on the left shows no immunoreactivity of flt-1 in endothelial cells (−arrow). The photograph on the right shows strong immunoreactivity (indicated by red staining) of flt-1 in endothelial cells (+arrow). Original magnification 20×.

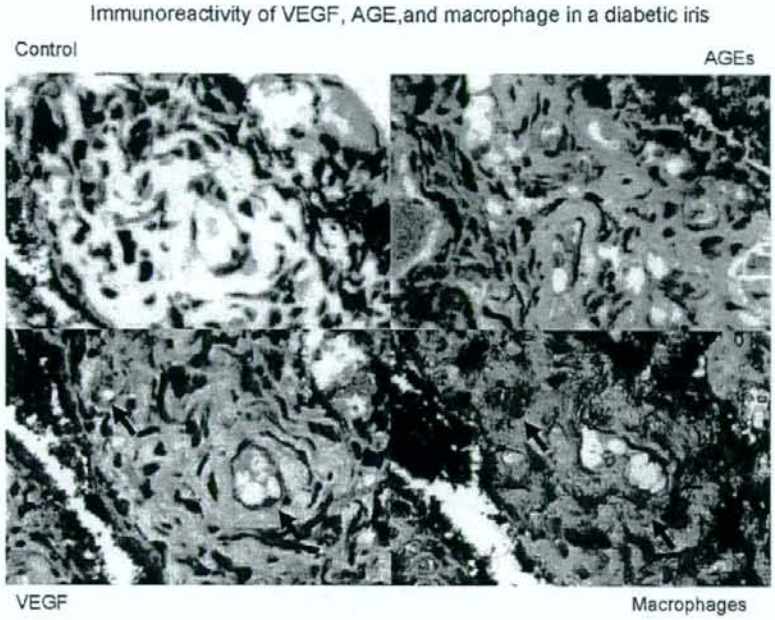

Fig. 10 – Immunoreactivity of VEGF, AGEs, and macrophages in a diabetic iris. Upper left, BSA-negative control. Upper right, extensive staining of AGEs in extracellular substances (indicated by red staining). Lower left, extensive staining of VEGF in endothelial cells (arrows, red staining). Lower right, extensive staining of macrophages around micro-vessels (arrows, red staining). Original magnification 20×.

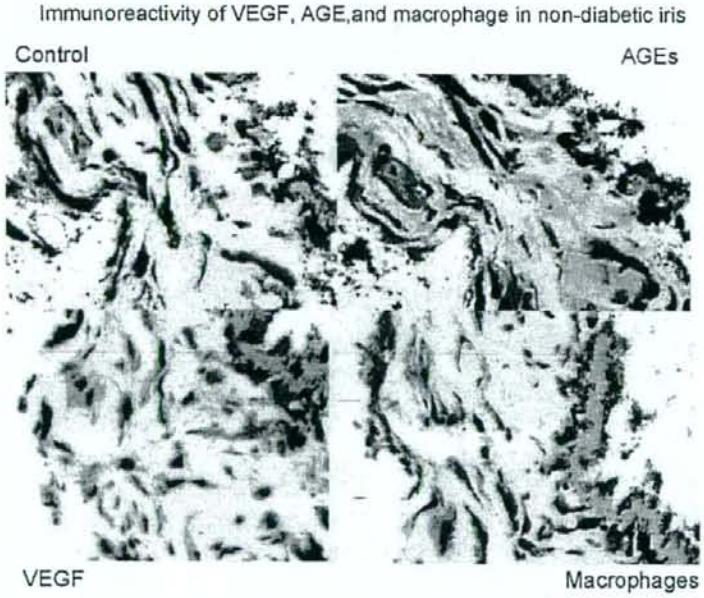

Fig. 11 – Immunoreactivity of VEGF, AGEs, and macrophages in a non-diabetic iris. Upper left, BSA-negative control. Upper right, extensive mild staining of AGEs in extracellular substances (indicated by red staining). Lower left, absence of staining of VEGF in endothelial cells. Lower right, absence of staining of macrophages around micro-vessels. Original magnification 20×.

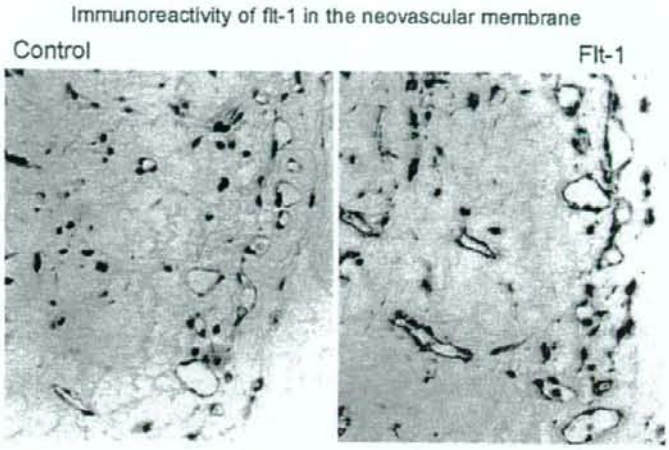

Fig. 12 – Immunoreactivity of flt-1 in neovascular membranes. Left, BSA-negative control shows no immunoreactivity of flt-1 in endothelial cells. Right, strong immunoreactivity of flt-1 in endothelial cells. Original magnification 20×.

However, high expression flt-1 in neovascular membranes strongly suggests that it contributes to the development of neovascular membranes in human diabetic retinopathy. From the result of the current study, flt-1 plays an important role in the development of retinal neovascular membranes, but the role is uncertain in the iris and retina.

AGE levels in both the aqueous humor and vitreous humor were higher in diabetic eyes than in non-diabetic eyes as

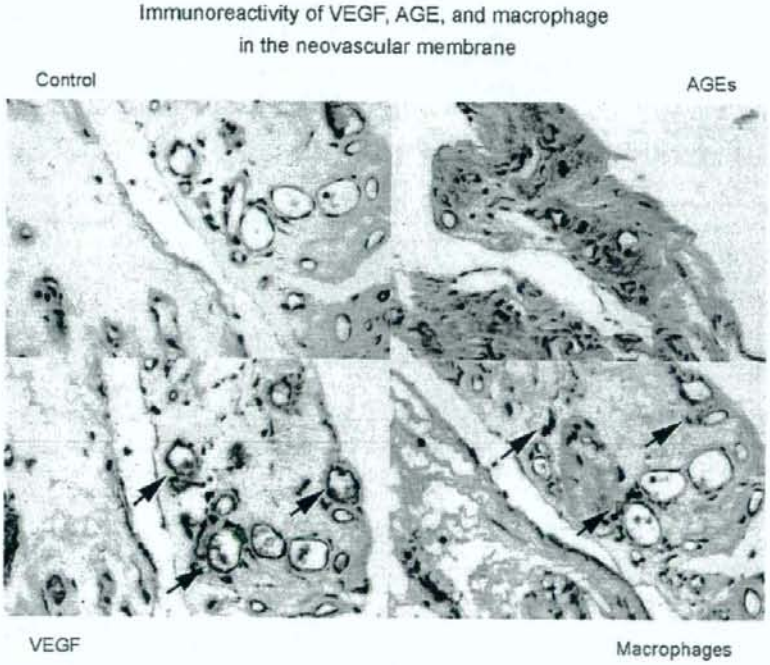

Fig. 13 – Immunoreactivity of VEGF, AGEs, and macrophages in a neovascular membranes. Upper left, BSA-negative control. Upper right, extensive staining of AGEs in extracellular substance (indicated by red staining). Lower left, extensive staining of VEGF in endothelial cells (arrows, indicated by red staining). Lower, extensive staining of macrophages around microvessels (arrows, indicated by red staining). Original magnification 20×.

reported previously [22,23,27]. However, although AGE levels in the vitreous humor were higher than in the aqueous humor, the AGE levels in the vitreous did not correlate with those in the aqueous humor in the current study. Furthermore, AGE levels also were not correlated with VEGF levels in either the aqueous humor or the vitreous humor, although some reports [27] showed a good correlation between the amounts of AGE and VEGF in the vitreous humor. We think that there are several reasons for this dissociation. VEGF is soluble and may be diluted in both aqueous and vitreous fluid, but most AGEs in the vitreous are stable in the vitreous gel or retinal neovascular membrane. AGE accumulate with aging and hyperglycemia and AGE accumulation cannot be reversed by glycemic control. This explanation does not contradict the Epidemiology of Diabetes Intervention and Complications Study [28] that showed that the original conventional treatment group did not prevent the progression of diabetic retinopathy by intensive treatment for 4 years as a mechanism of imprinting or hyperglycemic memory. On the other hand, expression of VEGF can be reduced by retinal photocoagulation [8].

The immunostaining of AGEs and VEGF showed that the former was present widely throughout the interstitial tissue but the latter was localized around macrophages. These findings were more prominent in neovascular membranes than in iris and showed that the major source of VEGF in diabetic eyes was the retina. Furthermore, once neovascular membrane had developed, AGE accumulation in the interstitial tissue of the membrane and macrophages around the new vessels may be the major stimulant and source, respectively, of VEGF production. Soluble AGEs in the aqueous and vitreous humor would not be expected to play as great a role in VEGF production as AGEs that have accumulated in the tissue.

From experimental studies in vitro and in vivo, VEGF and AGEs are theorized to play important roles in the development of diabetic retinopathy. The present clinical study, which analyzed VEGF, one of the VEGF receptors, and AGEs simultaneously in samples obtained from the same patients, supports previous studies [1,2,5–8,14,21,22,24], although the specimen sizes and types of analyses were limited, which is usually the case for this type of clinical study.

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Conflict of interest

The authors state that they have no conflict of interest.

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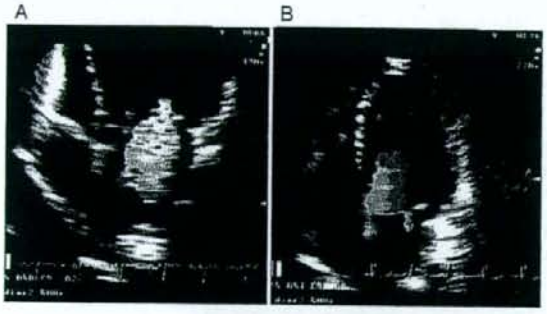

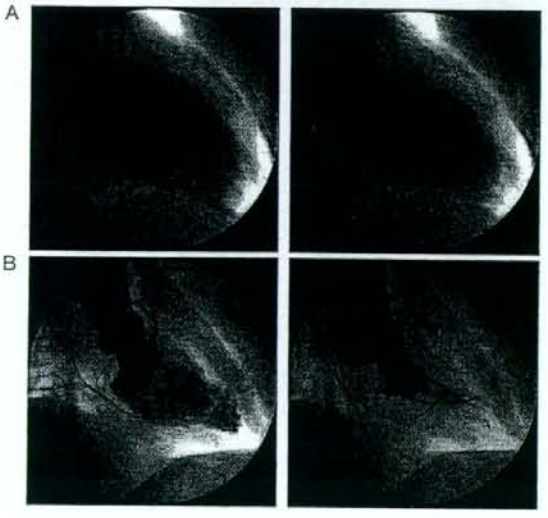

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Daily Exercise and Bone Marrow-Derived CD34+/133+ Cells After Myocardial Infarction Treated by Bare Metal Stent Implantation

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Background The aims of the present study were to explore the mobilization of bone marrow-derived CD34+/133+ cells in patients with acute myocardial infarction (AMI) and bare metal stent implantation who participated in daily exercise training, and associations with exercise capacity and restenosis.

Methods and Results Participants comprised 23 Japanese men with AMI (Killip 1) who had been treated with a bare metal stent. All patients were advised to walk for 30–60 min/day, at least 4 times per week starting at 11 days after AMI, and were instructed to record the amount of time spent walking each day. At 10 days and then at 3 months after onset of AMI, symptom-limited cardiopulmonary exercise tests were performed and the number of CD34+/133+ cells in the peripheral blood were measured by fluorescence-activated cell sorter analysis. At 3 months after AMI, the number of CD34+/133+ cells and oxygen consumption at anaerobic threshold were higher in the high exercise group (ie, exercise duration >4 h/week) than the low exercise group (ie, exercise duration <2 h/week). At 3 months after AMI, the number of CD34+/133+ cells significantly correlated with oxygen consumption at the anaerobic threshold (p=0.002).

Conclusion Moderate daily exercise of >4 h/week increases exercise capacity and the number of circulating CD34+/133+ cells at 3 months after AMI. (*Circ J* 2008; **72:** 897–901)

Key Words: Endothelial progenitor cell; Exercise; Myocardial infarction

The available evidence indicates that bone marrow-derived endothelial progenitor cells (EPC) can have beneficial effects on vascular repair and angiogenesis, and can protect against atherosclerosis[1,2] In a recent study using a non-human primate model, we found that transplanted bone marrow-derived CD34+ stem cells mediated improvement of perfusion and contraction in infarcted myocardium[3] Reports indicate that intracoronary administration of bone marrow CD34+ cells improves recovery of the left ventricular (LV) contractile function in patients with reperfused acute myocardial infarction (AMI)[4–6] Inoue et al recently reported that after bare-metal stenting, levels of CD34+ cells increased, reaching a maximum 7 days after stenting; and that this effect was more marked in patients with restenosis than in those without restenosis. These findings suggest that CD34+ bone marrow-derived cells can also differentiate into vascular smooth muscle cells, possibly leading to neointimal thickening and restenosis, which are associated with inflammation in post-stent local vascular injury[7] Shintani et al have reported that levels of EPC, which express CD34 and vascular endothelial growth factor receptor (VEGF) 2, increased transiently in patients with AMI[8] Exercise training and physical activity play an important role in the prevention and treatment of ischemic coronary heart disease, including AMI[9,10] Recent reports indicate that exercise training can increase the number of circulating EPC in animal models and humans with angina pectoris[11–13] Yet, little is known about the relationship between EPC mobilization and daily exercise several months after onset of AMI.

The purpose of the present study was to explore how the mobilization of CD34/CD133+ cells in peripheral blood is related to daily exercise training, exercise capacity and restenosis after bare metal stent implantation in patients with AMI.

Methods

Study Population

This was a single-center prospective case series study. The study population included 23 patients with AMI, who were admitted to our CCU between May and November 2004, and who received successful reperfusion therapy with bare metal stents within 12 h following the onset of AMI. Exclusion criteria were: Killip grade 2 or more; residual stenosis; LV ejection fraction <40%; patients on hemodialysis; and age >80 years. Female patients were excluded because they are less effective than male patients in cardiac rehabilitation, and have the potential of confounding the effects on EPC kinetics that occur during the menstrual cycle[14,15] All

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Table 1 Baseline Characteristics in Patients Grouped According to Amount of Time Spent Exercising

	Group 1 (n=10)	Group 2 (n=6)	Group 3 (n=7)	p value
Age (years)	59±10	61±6	61±7	0.834
BMI	24.1±3.1	23.9±2.8	25.2±1.5	0.608
LVEF (%)	48±13	48±7	53±12	0.672
Max CK (mg/dl)	3,515±2,520	2,716±3,244	1,435±532	0.226
Anterior MI	7 (70%)	2 (33%)	5 (57%)	0.358
HbA_{1c} (%)	5.6±0.8	5.3±0.3	5.9±1.9	0.641
LDL-C (mg/dl)	100±18	114±18	89±11	0.047**
CRP (mg/dl)*	0.44±0.38	0.52±0.64	0.54±0.65	0.934
BNP (pg/ml)*	125±101	92±74	93±43	0.641
Smoker	2 (20%)	1 (17%)	2 (29%)	0.860
Peak $\dot{V}O_2$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	19.5±3.6	20.3±5.7	22.3±3.0	0.390
$\dot{V}O_2$ at AT ($ml \cdot kg^{-1} \cdot min^{-1}$)	11.4±1.8	12.4±2.8	13.6±1.7	0.130
Medication at discharge				
ACEI	6 (60%)	4 (66%)	5 (71%)	0.884
ARB	3 (30%)	2 (33%)	2 (28%)	0.982
β-blocker	7 (70%)	3 (50%)	3 (43%)	0.503
Statin	10 (100%)	5 (83%)	7 (100%)	0.227
Aspirin	10 (100%)	6 (100%)	7 (100%)	
Ticropidine	10 (100%)	6 (100%)	7 (100%)	
$CD34^+/133^+$ cells/100 μl*	72±40	94±67	87±36	0.619

Values are the mean±SD.

**Sampled at 10 days after onset of acute myocardial infarction (AMI). Patients were divided into 3 groups, according to amount of time spent exercising (group 1: 0–119 min/week, group 2: 120–239 min/week, group 3: ≥240 min/week).*

***p=0.014 between groups 2 and 3.*

BMI, body mass index; LVEF, left ventricular ejection fraction; CK, creatine kinase; MI, myocardial infarction; HbA_{1c}, hemoglobin A_{1c}; LDL-C, low-density lipoprotein-cholesterol; CRP, C-reactive protein; BNP, brain natriuretic peptide; AT, anaerobic threshold; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.

patients gave written informed consent to the study protocol, which was approved by the Ethics Committee of Jichi Medical University.

Protocol

Patients were instructed to walk 30–60 min per day, 4 times per week (ie, 120–240 min/week),[6,17] starting at 11 days after AMI and stent implantation, and to record in their exercise diary the duration of time spent walking.

Cardiopulmonary Exercise Stress Test (CPX) At 10 days and then at 3 months after AMI, all patients underwent a symptom-limited CPX between 14:00 and 15:00 after a fasting period of at least 5 h in order to measure oxygen consumption at the anaerobic threshold and at peak oxygen consumption (peak $\dot{V}O_2$), using an electronically braked cycle ergometer (Ergometer 2320®, Minato Medical Science Co, Ltd, Osaka, Japan) at a constant rate of 60 beats/min in the upright position, as described elsewhere![6] During the exercise test, blood pressure and 12-lead ECG were monitored. The work rate was increased using a 15 W/min ramp until the onset of chest pain, dyspnea, leg fatigue, or significant ST depression. Expired air gas was analyzed continuously using a metabolic cart (AE-300SR; Minato Medical Science Co Ltd). The anaerobic threshold was found using the V-slope method by determining the starting point of the non-linear increase in carbon dioxide output![6–18]

Blood Sampling and Assay Fasting blood samples were taken from the cubitus vein while the patient was at rest, just before the exercise test, at 10 days and at 3 months after AMI. Trained technicians, blinded to the patients' information, measured the number of $CD34^+/45^+/133^+$ cells, using fluorescence-activated cell sorting (FACS) (FACSCalibur™; BD Biosciences, San Jose, CA, USA) with the following antibodies: anti-human $CD34^+$, $CD45^+$ and $CD133^+$ (BD Biosciences). After gating $CD45^+$ (a marker of nuclear cells) cells from whole blood, we measured the number of cells that were double-positive for CD34 and CD133. We also measured levels of highly sensitive C-reactive protein (hCRP), hemoglobin A_{1c} (HbA_{1c}), low-density lipoprotein-cholesterol and brain natriuretic peptide (BNP). The hCRP was measured by a monoclonal antibody using latex that allows the measurement of samples with low (0.01 mg/dl) to high (42 mg/dl) concentrations (NANOPIA CRP; Daiichi Pure Chemical Co, Ltd, Tokyo, Japan). The normal value of C-reactive protein is <0.2 mg/dl. BNP was determined using a radioimmunoassay kit (Shionogi Pharmaceuticals Co, Osaka, Japan). Interleukin-6 levels at 3 months after AMI were measured using an enzyme-linked immunosorbent assay kit (R&D Systems Inc, Minneapolis, MN, USA and Otsuka Pharmaceutical Co, Osaka, Japan). Angiographic restenosis (late luminal loss) was analyzed at 6 months after AMI by performing a quantitative coronary angiography (Advantex LC/LP+; GE Yokogawa Medical Systems, Tokyo, Japan).

Statistical Analysis

Data are expressed as the mean±SD. Mean values were compared among the 3 groups by analysis of variance, followed by Scheffe's multiple comparison test. Categorical variables are described as frequencies and were compared using chi-square analysis. Correlations were assessed using Fisher's coefficient (r). Probability values of $p<0.05$ were considered to indicate statistical significance. All statistical analysis was performed using StatView 5.0 software (SAS institute, Cary, NC, USA).

Results

Only 13 of the 23 patients performed walking training according to our instructions (ie, walking >2 h/week) after discharge. Patients were divided into 3 groups, according to duration of time spent exercising: Group 1, 0–119 min/week;

Table 2 Characteristics at 3 Months After AMI in Patients Grouped According to Amount of Time Spent Exercising

	Group 1 (n=10)	Group 2 (n=6)	Group 3 (n=7)	p value
Exercise duration/week (min)	61±30	163±40	474±229	<0.001*
BMI	23.9±2.9	23.1±2.5	24.2±2.6	0.755
LVEF (%)	51±10	53±16	55±8	0.851
HbA_{1c} (%)	5.4±0.5	5.4±0.1	5.4±0.6	0.998
LDL-C (mg/dl)	91±31	87±19	83±19	0.849
CRP (mg/dl)	0.91±2.4	0.28±0.5	0.2±0.27	0.634
BNP (pg/ml)	77±73	49±32	33±18	0.251
IL-6 (pg/ml)	3.27±4.63	1.41±1.01	1.31±0.66	0.401
Smoker at 3 months	0 (0%)	0 (0%)	0 (0%)	
Peak $\dot{V}O_2$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	21.1±6.4	26±3.2	23.7±3.4	0.180
AT ($ml \cdot kg^{-1} \cdot min^{-1}$)	12.1±2.9	13.8±1.8	15.3±2.3	0.055**
Medication at 3 months				
ACEI	6 (60%)	4 (66%)	5 (71%)	0.884
ARB	3 (30%)	2 (33%)	2 (28%)	0.982
β-blocker	7 (70%)	3 (50%)	3 (43%)	0.503
Statin	10 (100%)	5 (83%)	7 (100%)	0.227
Aspirin	10 (100%)	6 (100%)	7 (100%)	
Ticropidine	2 (20%)	1 (17%)	2 (28%)	0.86
CD34+/133+ cells/100 μl	51±26	92±49	101±51	0.048††

*p<0.01 between groups 1 and 3, and between groups 2 and 3.
**p=0.005 between Group groups 1 and 3.
††p=0.023 between groups 1 and 3.
Values are the mean±SD. IL-6, interleukin-6. See Table 1 for all other abbreviations.

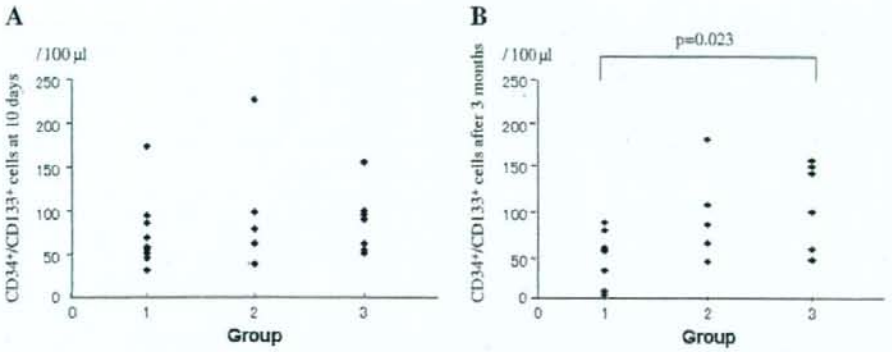

Fig 1. Number of CD34+/133+ cells (A) 10 days after acute myocardial infarction (AMI) and (B) 3 months after AMI in each patient group. Group 1 (n=10) exercised <120 min/week, group 2 (n=6) exercised between 120 and 239 min/week, and group 3 (n=7) exercised >240 min/week.

group 2, 120–239 min/week; group 3, >240 min/week. Table 1 shows the baseline clinical characteristics of each group. During the study period, there were no deaths from any cause, no symptoms of angina pectoris, and no orthopedic problems. Table 2 shows the characteristics of each group at 3 months after AMI. Oxygen consumption at the anaerobic threshold at 3 months was higher in group 3 (15.3±2.3 $ml \cdot kg^{-1} \cdot min^{-1}$) than in group 1 (12.1±2.9 $ml \cdot kg^{-1} \cdot min^{-1}$) (p=0.05). The number of CD34+/133+ cells at 3 months was greater in group 3 (101±51/100 μl) than in group 1 (51±26/100 μl) (p=0.023), although there was no significant difference at 10 days after AMI (Fig 1). Delta CD34+/133+ cell numbers between 10 days and 3 months after AMI onset significantly increased in group 3 compared with in group 1 (Fig 2; p=0.049). At 3 months after AMI, the number of CD34+/133+ cells correlated significantly with oxygen consumption at the anaerobic threshold (p=0.001) (Fig 3).

Late luminal loss was not statistically significant among the patient groups. The correlation between late luminal loss and the number of CD34+/133+ cells was almost significant at 10 days after AMI (p=0.06; data not shown), but there was no correlation between late luminal loss and the number of CD34+/133+ cells at 3 months.

Discussion

The present study has reported how mobilization of CD34+/CD133+ cells in peripheral blood is related to daily exercise training, exercise capacity and restenosis after bare metal stent implantation in patients with AMI. Exercise capacity and the number of circulating CD34+/133+ cells were greater for patients who walked >4 h/week than for those who walked <2 h/week, and there was a positive correlation between exercise capacity and the number of circulating CD34+/133+ cells.

The quality and quantity of EPC reflect endothelial function? An increase in the number of circulating CD34+/133+ cells in the high exercise group may contribute to moderate improvement of endothelial function, reduction of periph-