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Atheroprotective Roles of Adiponectin via CCL2 Inhibition

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Aim: Adiponectin (APN) exhibits different atheroprotective effects, and we have previously reported that APN function is modulated by its binding proteins, E-selectin ligand 1, Mac-2 binding protein, and cystatin C. In the present study, we aimed to identify a novel atheroprotective mechanism of APN via C–C motif chemokine 2 (CCL2).

Methods: We conducted iMAP®-intravascular ultrasound (IVUS) in 111 Japanese male patients with stable angina. The plaque characteristics were determined where "plaque burden" [(EEM CSA – lumen CSA)/(EEM CSA) × 100 (%)] >50%, and their correlation with serum CCL2 and APN levels was analyzed. Using western blot analysis, the effects of APN on the biological effects of CCL2 were examined in their mutual binding by co-immunoprecipitation assay, the monocyte migration, and the phosphorylation of MAP kinases.

Results: In a clinical study, we found that the percentage of plaque in the culprit lesion was correlated positively with serum CCL2 and negatively with serum APN levels, with significance. We identified CCL2 as a novel APN-binding serum protein using immunoprecipitation and western blot analysis. CCL2-induced phosphorylation of MAP kinases and monocyte migration was significantly attenuated by APN *in vitro*.

Conclusion: The opposite association of APN and CCL2 on the percentage of coronary plaque might be caused by their direct interaction and competitive functions on monocyte migration.

Key words: Adiponectin, CCL2, Monocyte, MAP kinase

Abbreviations: APN, adiponectin; CAD, coronary artery disease; CVD, cardiovascular diseases; IVUS, intravascular ultrasound; EEM, external elastic membrane; CSA, cross-sectional areas

Introduction

Cardiovascular diseases (CVDs) are still among the leading causes of death in many countries, and their major cause is atherosclerosis. Atherosclerosis is regarded as a chronic inflammatory condition in which abundant immune-competent cells produce cytokines in the lesions, especially proinflammatory cytokines[1, 2]. The first step in atherosclerosis is endothelial inflammation with enhanced expression of adhesion molecules, which allows mononuclear leukocytes (such as monocytes and T cells) to attach to the endothelium and penetrate the intima. As atherosclerosis progresses, monocyte-derived macrophages and the synthetic phenotype of smooth muscle cells phagocytose oxidized low-density lipoprotein and transform into foam cells. The lipid-laden foam cells degrade the extracellular matrix to establish vulnerable plaque[3]. Prothrombotic materials are exposed to the coagulation system when plaques are damaged and rupture, which leads to the development of acute coronary syndrome[4]. Therefore, evaluating the vulnerability of plaque is crucial, and several modalities, including coronary angiography,

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coronary computed tomography angiography, optical coherence tomography, and intravascular ultrasound (IVUS), have been invented for this purpose[5].

IVUS is a very useful modality for evaluating the severity of CVD, as it accurately displays direct images of the inside of the vessel as well as plaque morphology and vessel composition[6, 7]. An evolved version of IVUS, iMAP®-IVUS, allows for the real-time quantification of coronary plaques, categorizing them into four different subtypes (i.e., fibrotic, lipidic, necrotic, and calcified). The plaques that contain many lipidic and necrotic components are regarded as "vulnerable," with a high risk of plaque rupture. However, IVUS has the disadvantage of being invasive, and thus a non-invasive and simpler method has been explored for evaluating plaque characteristics.

Adiponectin (APN) is an adipocytokine that is secreted mainly from adipocytes[8, 9]. APN has various forms *in vivo*, including trimer, hexamer, and high molecular forms, and it is well known to be associated with metabolic syndrome and atherosclerosis. Hypoadiponectinemia plays a central role in obesity-related diseases, including insulin resistance/type 2 diabetes, hypertension, and CVD. Studies of diverse populations have demonstrated a negative correlation between serum APN levels and the prevalence and extent of coronary artery disease (CAD)[10-14]. In addition, high plasma APN concentrations are associated with a reduced risk of acute myocardial infarction in men over a long-term follow-up[15]. High-molecular-weight APN, the major active form, appears to be selectively reduced in the presence of CAD[16]; however, the mechanisms underlying its atheroprotective effects have not been fully elucidated.

The main features of atherosclerosis are the continuous immigration and infiltration of activated macrophages and T cells into the atherosclerotic lesions, and these cells are recruited to the lesions via the guidance of endothelial leukocyte adhesion molecules and chemoattractants[17]. Chemokines constitute a structurally related family of chemotactic cytokines and are expressed by activated endothelial cells, smooth muscle cells, and emigrated leukocytes. Different chemokines are reported to be involved in the pathogenesis of atherosclerosis[18, 19]. The C–C motif chemokine ligand 2 (CCL2), known as monocyte chemoattractant protein-1 (MCP-1), belongs to the CC family of the inflammatory chemokines and acts on monocytes, T cells, and NK cells through the interaction with its receptor, the C–C chemokine receptor 2 (CCR2). CCL2 induces monocyte migration and macrophage infiltration into the arterial wall, and recent studies have shown that the lack of CCR2 in apolipoprotein E-null mice and the deletion of the MCP-1 gene in transgenic mice expressing human apolipoprotein B resulted in a decrease in the formation of atherosclerosis lesions[20, 21]. These results suggested that the monocyte migration and macrophage infiltration induced by CCL2 play essential roles in the initiation of atherosclerosis.

Recently, we determined several APN-interacting molecules, E-selectin ligand-1 (ESL-1)[22], cystatin C (CysC)[23], and Mac-2 binding protein (M2BP)[24], and we demonstrated that the functions of APN and its interacting molecules are mutually regulated. Moreover, the possible interaction between APN and several chemokines was suggested in a previous report[25]. On the basis of these reports and the opposite effects of APN and CCL2 on atherosclerosis, we hypothesized a direct interaction between APN and CCL2. In the present study, we examined the relationship between coronary plaque parameters with APN or CCL2 in patients with stable angina and the roles of the interaction between APN and CCL2 on atherosclerosis *in vitro*.

Methods

Patients

In the present study, we enrolled consecutive 111 male patients with stable angina who underwent elective percutaneous coronary intervention and iMAP®-IVUS (Boston Scientific, Marlborough, MA) at the Department of Cardiology in Hyogo Prefectural Nishinomiya Hospital, Japan. Patients who were aged >82 years, had renal dysfunction (serum creatinine >1.5 mg/dL) or malignant disease, or whose target lesions were chronic total occlusion or in-stent restenosis were excluded from this study. This study was conducted according to the Declaration of Helsinki and was approved by the ethics committees of Hyogo Prefectural Nishinomiya Hospital and Osaka University Graduate School of Medicine.

Measurement of Serum Parameters

Fasting serum biochemical parameters were measured in commercial laboratories. Serum APN levels and CCL2 were measured using a total APN enzyme-linked immunosorbent assay (ELISA) kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and a human CCL2/MCP-1 immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA), respectively, according to the manufacturer's instruction.

Evaluation of Plaque in Coronary Arteries by iMAP®-IVUS

IVUS was conducted with a 40 MHz catheter (Atlantis ™ SR Pro Coronary Imaging Catheter,

Boston Scientific, Marlborough, MA), and the external elastic membrane cross-sectional areas (EEM CSA) and lumen CSA were measured at each section of PCI target lesions. We examined the plaque characteristics where "plaque burden" [(EEM CSA – lumen CSA)/(EEM CSA) × 100 (%)] > 50%. The percentage of plaque (%Plaque) was calculated from the integration using both the plaque and the EEM CSA of each section and was defined as "Σ (EEM CSA – lumen CSA)/Σ (EEM CSA) × 100 (%)." The plaques were automatically classified into four histological tissue types, according to the tissue components (fibrotic, lipidic, necrotic, or calcified), and the percentage of each component in the entire plaque was calculated as "Σ (each component CSA)/Σ (EEM CSA – lumen CSA) × 100 (%)" and named as %Fibrotic, %Lipidic, %Necrotic, or %Calcified, respectively.

Plasmids

Myc-tagged APN (myc-APN) expression vector was generated by incorporating the human APN cDNA sequence into the upstream of myc in the pSecTag2 vector (Invitrogen ™, Carlsbad, CA, USA). FLAG-tagged CCL2 (FLAG-CCL2) expression vector was generated by adding 3xFLAG tags to the signal peptide-deleted human CCL2 cDNA in the pSF-CMV-NEO-NH2-PPT-3xFLAG vector (Sigma-Aldrich, St. Louis, MO, USA). We aimed to use these two vectors for protein secretion.

Cell Culture

Human embryonic kidney cells 293T (HEK293T) were cultured in Dulbecco's modified Eagle medium (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS; Biowest, NW Business Park Lane Riverside, MO, USA), 0.1 mg/mL streptomycin, and 100 U/mL penicillin G (SM/PG; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The monocytic cell line, THP-1, was purchased from the Riken BioResource Research Center (Ibaraki, Japan) and cultured in RPMI 1640 (Nacalai Tesque, Kyoto, Japan) with 10% FBS and SM/PG. Cell culture dishes were maintained in a humidified incubator at 37℃ and 5% CO_2.

Transfection

For *in vitro* analysis, myc-APN and FLAG-CCL2 expression vectors were co-transfected into HEK293T cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's protocol.

Immunoprecipitation

For *in vivo* analysis, first, we prepared anti-APN antibody-conjugated magnetic beads by coupling 100 μg mouse anti-human APN monoclonal antibody (ANOC9121; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) to 5 mg tosylactivated Dynabeads (M-280; Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. Thereafter, 0.25 mg of these magnetic beads were used for the immunoprecipitation of human serum containing 200 ng APN. For *in vitro* analysis, the supernatant of the cell culture of HEK293T cells overexpressing myc-APN and FLAG-CCL2 was used for immunoprecipitation with anti-c-myc magnetic beads (Thermo Fisher Scientific, Waltham, MA, USA) or anti-FLAG magnetic beads (MBL, Nagoya, Japan).

Immunoblotting

Immunoprecipitated samples were used for gel electrophoresis and transferred to the nitrocellulose membranes (BioRad, Hercules, CA, USA). For the *in vivo* analysis, the membranes were sequentially incubated with a biotinylated mouse anti-human APN antibody and an HRP-conjugated streptavidin (Thermo Fisher Scientific, Waltham, MA, USA) or with a mouse anti-human CCL2 monoclonal antibody (R&D Systems, Inc., Minneapolis, MN, USA) and an HRP-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, Inc., West Grove, PA, USA). The secondary antibody was detected by enhanced chemiluminescence systems (Thermo Fisher Scientific, Waltham, MA, USA). For the *in vitro* analysis, the membranes were incubated with an HRP-conjugated anti-c-myc monoclonal antibody (Wako, Osaka, Japan) or an HRP-conjugated anti-FLAG monoclonal antibody (MBL, Nagoya, Japan), and these antibodies were detected by enhanced chemiluminescence systems.

Migration Assay

Using the CytoSelect ™ 96-Well Cell Migration Assay kit (Cell Biolabs, Inc., San Diego, CA, USA), the migration of THP-1 cells was determined, in accordance with the manufacturer's instruction. THP-1 cells were cultured overnight in RPMI1640 containing 0.5% FBS with or without 10 μg/mL APN (Oriental Yeast Co., LTD., Tokyo, Japan). After adjusting the cell density to 2×10^6 cells/mL in serum-free RPMI1640 with or without 10 μg/mL APN, 2×10^5 cells were added to the upper chamber. Serum-free RPMI1640 with or without 200 ng/mL CCL2 (R&D Systems, Inc., Minneapolis, MN, USA) was added to the lower well. The cells that passed through the membrane were collected and counted after 2 h of

Table 1. Clinical and plaque characteristics of the enrolled patients

	mean ± SD	range
Age (year)	70 ± 10	37–88
BMI (kg/m^2)	24.6 ± 3.3	16.0–34.5
Systolic blood pressure (mmHg)	134 ± 17	92–171
Diastolic blood pressure (mmHg)	78 ± 12	56–104
Fasting plasma glucose (mmol/L)	6.11 ± 1.89	4.00–13.60
HbA1c (%)	6.5 ± 1.1	4.9–10.2
Total cholesterol (mg/dL)	167.6 ± 34.0	106–310
Triglycerides (mg/dL)	144.4 ± 84.5	37–465
HDL-C (mg/dL)	41.7 ± 9.3	23–80
cLDL-C (mg/dL)	98.2 ± 28.2	46–210
hsCRP (mg/L)	3.06 ± 6.10	< 0.10–43.40
eGFR (mL/min/ 1.73 m^2)	65.9 ± 18.9	27.3–127.9
APN (mg/mL)	10.4 ± 5.9	2.5–34.2
CCL2 (pg/mL)	240.8 ± 169.0	73.8–1716.9
IVUS		
%Plaque	66.4 ± 5.4	55.1–78.7
%Fibrotic	53.2 ± 12.1	22.1–86.6
%Lipidic	11.7 ± 2.9	4.5–20.2
%Necrotic	32.4 ± 10.2	8.6–58.2
%Calcified	3.2 ± 2.4	0.2–11.8
%Lipidic plus Necrotic	44.0 ± 11.6	13.1–74.6

DATA are expressed as mean ± standard deviation (SD).
cLDL-C, calculated LDL-C; hsCRP, high sensitive C-reactive protein

incubation at 37℃.

Quantification of Phosphorylated and Total MAP Kinases

The THP-1 cells were stimulated for 1 min with 100 ng/mL CCL2 with or without 1.5 μg/mL APN after 24 h of serum starvation in RPMI1640 containing 0.1% BSA. The cells were lysed in Laemmli buffer, and the cell lysates were used for immunoblotting with the antibodies against ERK (Abcam, Cambridge, UK), phospho-ERK (Cell Signaling Technology (CST), Danvers, MA, USA), JNK (CST), phospho-JNK (CST), p38-MAPK (Bethyl Laboratories, Inc., Montgomery, USA), or phospho-p38-MAPK (CST). Using Image J (National Institutes of Health, Bethesda, MD, USA), the bands' intensity was evaluated.

Statistical Analysis

Statistical analysis was conducted with JMP Pro version 14.0.0 (SAS Institute Inc., Cary, NC, USA). To evaluate the relationship between the plaque parameters and clinical characteristics, Spearman's correlation coefficient was used. The Student *t*-test was used to compare two independent groups. *P* values of <0.05 are considered statistically significant.

Table 2. Medication of the patients

	number (%)
Anti-diabetic agents	46 (41.4%)
Insulin only	7 (6.3%)
Oral hypoglycemic agents (OHA) only	30 (27.0%)
Insulin + OHA	9 (8.1%)
Anti-hypertensive agents	81 (73.0%)

Results

Patient Characteristics

Tables 1 and 2 list the clinical characteristics and medication of the 111 male CAD patients. The average age and body mass index (BMI) was 70 ± 10 years and 24.6 ± 3.3 kg/m^2, respectively. All patients were diagnosed with stable angina and received medication for the secondary prevention of CVD. Insulin and/or oral hypoglycemic agents and anti-hypertensive agents were used by 46 patients (41.4%) and 81 patients (73.0%), respectively. Because of the medical treatment, blood sugar levels were well controlled, and mean HbA1c levels were 6.5% ± 1.1%. Blood pressure was mildly controlled, and mean systolic and diastolic blood pressure was 134 ± 17

Table 3. Correlation between serum APN, CCL2 levels and clinical parameters or plaque characteristics

	APN		CCL2	
	ρ	p	ρ	p
Age	0.246	<0.01	0.178	0.062
BMI	−0.363	<0.0001	0.145	n.s.
Systolic blood pressure	0.199	n.s.	0.061	n.s.
Diastolic blood pressure	−0.125	n.s.	−0.175	n.s.
Fasting plasma glucose	−0.104	n.s.	−0.032	n.s.
HbA1c	−0.002	n.s.	0.180	0.059
Total cholesterol	−0.086	n.s.	−0.027	n.s.
Triglycerides	−0.418	<0.0001	0.170	n.s.
HDL-C	0.139	n.s.	−0.236	<0.05
cLDL-C	−0.045	n.s.	−0.068	n.s.
eGFR	0.002	n.s.	−0.028	n.s.
hsCRP	−0.129	n.s.	0.112	n.s.
CCL2	−0.111	n.s.		
IVUS				
%Plaque	−0.280	<0.005	0.277	<0.005
%Fibrotic	−0.096	n.s.	−0.074	n.s.
%Lipidic	0.059	n.s.	0.051	n.s.
%Necrotic	0.089	n.s.	0.082	n.s.
%Calcified	0.072	n.s.	0.014	n.s.
%Lipidic plus Necrotic	0.068	n.s.	0.089	n.s.

cLDL-C, calculated LDL-C; hsCRP, high sensitive C-reactive protein

mmHg and 78 ± 12 mmHg, respectively. The mean calculated LDL-C (cLDL-C) levels were 98.2 ± 28.2 mg/dL. The average serum APN and CCL2 levels were 10.4 ± 5.9 μg/mL and 240.8 ± 169.0 pg/mL, respectively. **Table 1** also shows the coronary plaque characteristics evaluated by iMAP®-IVUS, the percentage of plaque within the examined segments of the culprit lesions (%Plaque), and the percentage contribution of four histological tissue types to the plaque (%Fibrotic, %Lipidic, %Necrotic, and %Calcified). The average %Plaque was 66.4% ± 5.4%. The average %Fibrotic, %Lipidic, %Necrotic, %Calcified, and %Lipidic plus Necrotic were 53.2% ± 12.1%, 11.7% ± 2.9%, 32.4% ± 10.2%, 3.2% ± 2.4%, and 44.0% ± 11.6%, respectively.

Serum APN and CCL2 Levels Are Associated with Plaque Percentage, But not with Plaque Components

Next, we examined the clinical significance of APN and CCL2 with coronary plaques **(Table 3)**. Serum APN levels were correlated positively with age ($\rho = 0.246$, $p < 0.01$) and negatively with both BMI ($\rho = -0.363$, $p < 0.0001$) and TG ($\rho = -0.418$, $p < 0.0001$) with significance. Serum CCL2 levels had a negative correlation with HDL-C significantly ($\rho = -0.236$, $p < 0.05$). The percentage of plaque (%Plaque) was significantly correlated negatively with serum APN ($\rho = -0.280$, $p < 0.005$) and positively with serum CCL2 ($\rho = 0.277$, $p < 0.005$). However, neither serum APN nor CCL2 levels were associated with any histological plaque component.

CCL2 Binds to APN *in vivo* and *in vitro*

Masaie *et al.* examined the interaction between Immunoglobulin (Ig)-tagged chemokines and adiponectin *in vitro*[25]. They found that the globular adiponectin was able to bind to Ig-tagged SDF-1 and CCF18 and also possibly to Ig-tagged RANTES, or MCP-1. Accordingly, we examined the interaction between serum CCL2 and APN *in vivo* **(Fig. 1A)**. The immunoprecipitation with anti-APN antibody followed by the immunoblot with anti-CCL2 antibody demonstrated that human serum CCL2 was co-immunoprecipitated with APN. Next, we examined the CCL2–APN interaction *in vitro* using HEK293T cells expressing myc-APN and FLAG-CCL2. The immunoprecipitation with anti-myc antibody followed by the immunoblot with anti-FLAG antibody demonstrated that FLAG-CCL2 was co-immunoprecipitated with myc-APN **(Fig. 1B)**.

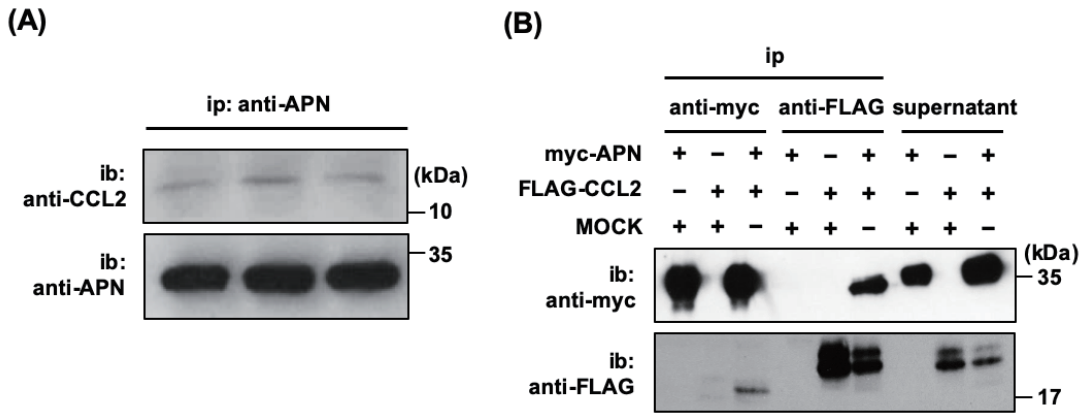

Fig. 1. CCL2 binds with APN in the serum and *in vitro*

(A) Human serum was immunoprecipitated with the anti-APN antibody, followed by the immunoblot either with the anti-CCL2 (upper panel), or with the anti-APN (lower panel) antibody. $n=3$.
(B) The supernatant of the cultured HEK293T cells that overexpress both myc-APN and FLAG-CCL2 was used for immunoprecipitation with either anti-myc, or anti-FLAG antibody, followed by the immunoblot with anti-myc (upper panel), or with anti-FLAG antibody (lower panel).

APN Prevents CCL2-Induced THP-1 Cell Migration

CCL2 is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages. Then, we examined the effects of APN on the THP-1 cell migration induced by CCL2 using Boyden chamber system (Fig. 2). The migration of THP-1 cells was significantly stimulated in the presence of 200 ng/mL CCL2 in 2 h. This CCL2-stimulating migration of THP-1 cells was significantly reduced by the pretreatment of 10 μg/mL APN overnight.

APN Suppresses Phosphorylation of MAP Kinases Induced by CCL2

We examined the effects of APN on CCL2-induced phosphorylation of MAP kinases in THP-1 cells to elucidate mechanisms of the APN–CCL2 complex. After 1 min treatment with CCL2 at 100 ng/mL, the phosphorylation levels of ERK, JNK, and p38 MAP kinases were all upregulated significantly ($p<0.05$; Fig. 3). When 100 ng/mL of CCL2 was preincubated with 1.5 μg/mL of APN, all the upregulated phosphorylation of MAP kinases was significantly decreased ($p<0.05$).

Discussion

In the present study, we examined the roles of APN and CCL2 on atherosclerosis *in vivo* and *in vitro*. First, we found that the percentage of plaque in the coronary culprit lesions was negatively correlated with serum APN levels and positively correlated with serum CCL2 levels in male patients with stable angina. Second, we found direct binding of both APN and CCL2 in serum and *in vitro* experiments. The significance of APN–CCL2 binding was functionally demonstrated by the inhibition of THP-1 cell migration and MAP kinase phosphorylation, and these mechanisms could explain the clinical findings.

Several reports regarding the relationship between cardiovascular plaque characteristics and serum levels of APN or CCL2 exist. Sawada *et al.* reported that serum APN levels were negatively associated with the presence of vulnerable coronary thin-cap fibroatheroma in patients with stable angina[26]. Conversely, Otake *et al.* reported an inverse relationship between serum APN and necrotic core ratio in both culprit and non-culprit lesions in patients with ACS, but not in those with stable angina[27]. Fuchs *et al.* reported that serum CCL2 levels were positively correlated with necrotic core areas in both stable and unstable angina patients but not with plaque burden[28]. Conversely, Cheng *et al.* reported that serum CCL2 levels were positively associated with plaque burden in patients with acute myocardial infarction and unstable angina[29]. Although there are some discrepancies among those clinical studies, APN and CCL2 are generally considered to have opposite effects on plaque vulnerability.

Because APN and CCL2 had opposite effects on coronary plaque and atherosclerosis, we hypothesized their direct interaction. A previous report suggested the interaction between APN and several chemokines using an Ig-tagged chemokine-coated ELISA plate, an artificial system[25]. Thus, we here examined the protein–protein interaction using the immunoprecipitation-immunoblot

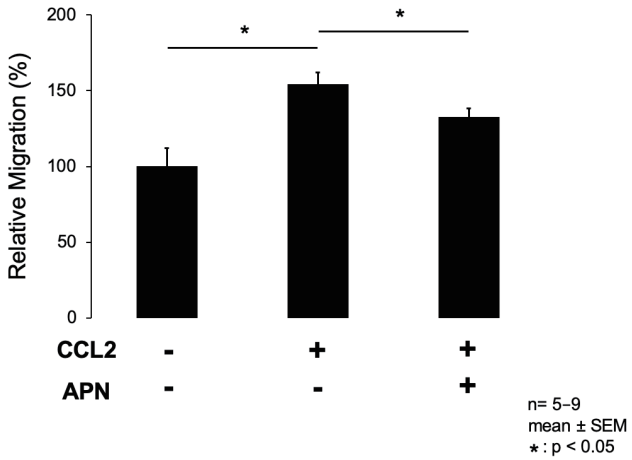

Fig. 2. APN inhibits CCL2-induced THP-1 cell migration

The migration of THP-1 cells was determined by a CytoSelect™ 96-Well Cell Migration Assay kit. THP-1 cells were cultured overnight in RPMI1640 containing 0.5% FBS with or without 10 μg/mL APN. On the day of analysis, those THP-1 cells were added to the upper chamber in serum-free RPMI1640 with or without APN, as well as the serum-free RPMI1640 with or without 200 ng/mL CCL2 in the lower chamber. The cells passing through the membrane during 2 h incubation at 37℃ were counted.
Data are expressed as mean ± SEM; $n = 5–9$, $^*p < 0.05$.

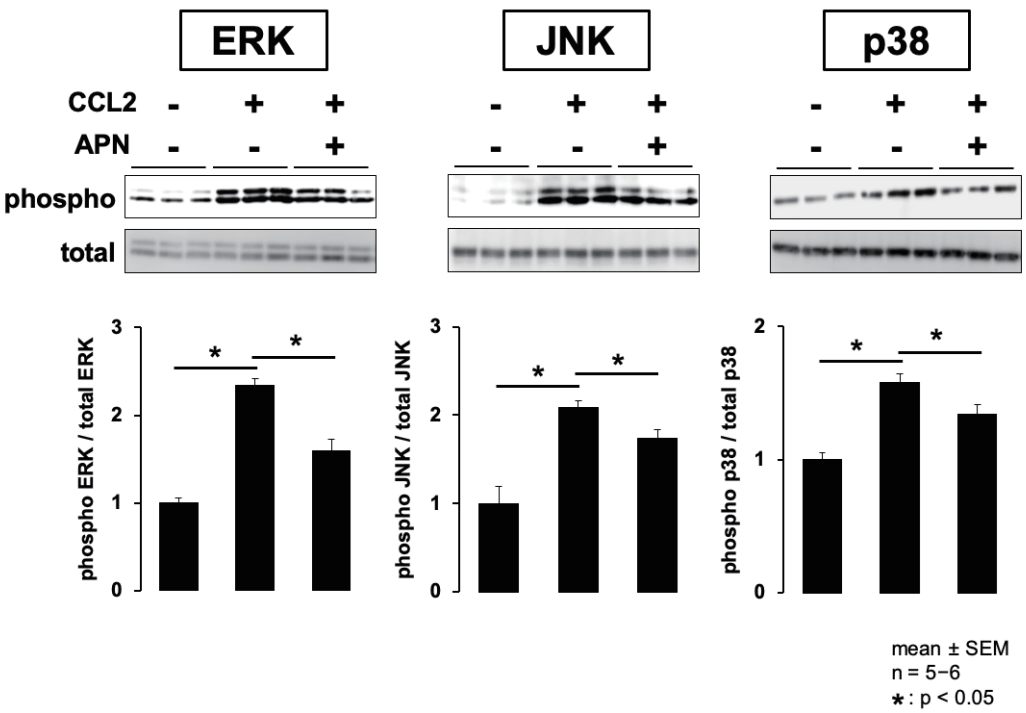

Fig. 3. APN prevents CCL2-induced MAP kinase activation in THP-1 cells

After 24 h serum starvation, the THP-1 cells were stimulated for 1 min with 100 ng/mL CCL2 with or without 1.5 μg/mL APN. Those cell lysates were analyzed by western blot using the antibodies against ERK and phospho-ERK (left lower panels), JNK and phospho-JNK (middle lower panels), and p38-MAPK and phospho-p38-MAPK (right lower panels). Representative figures are shown.
Data in the bar graphs (upper panels) are mean ± SEM of two independent measurements; $n = 5–6$, $^*p < 0.05$.

system.

First, we found CCL2 as a specific co-immunoprecipitated serum protein by anti-APN antibody, but because of the unavailability of a suitable anti-CCL2 antibody for immunoprecipitation, we failed to identify APN as a specific co-immunoprecipitant with CCL2 using the anti-CCL2 antibody. However, we found that CCL2 and APN were mutual co-immunoprecipitants in the immunoprecipitation and western blot analysis using epitope-tagged CCL2 and APN, indicating that the binding of APN to CCL2 was more conclusive. Interestingly, APN mainly bound to low-molecular-weight FLAG-CCL2 among the multiple forms in our observation, and the modification of CCL2 bound to APN has not been clarified yet. Therefore, further studies would be necessary to identify the precise binding mechanisms of these two molecules.

Next, we examined the effects of APN–CCL2 interaction on the biological roles of CCL2, *i.e.*, CCL2-induced chemotaxis of THP-1 cells. CCL2-induced chemotaxis of THP-1 cells was suppressed by APN at 1.5 μg/mL, the corresponding serum concentration of high-molecular-weight APN, which was a similar molar level of CCL2. Since CCL2/CCR2-induced MAPK activation (ERK1/2, p38/SAPK, and JNK) was previously reported in THP-1 monocytes[30], the rapid activation of MAPK in the present study is considered through the binding of CCL2 and CCR2. In addition, APN itself had no effects on MAPK activities (data not shown), suggesting that the inhibitory effects of APN on CCL2-induced rapid MAPK activation would be based on CCR2 inhibition by the binding of APN and CCL2. MAP kinases are necessary for proliferation, differentiation, apoptosis, and certain kinds of stress responses in various cells, including the activation of monocytes[31, 32]. Previous studies using specific inhibitors of MAP kinase pathways demonstrated that p38 and ERK play important roles in chemotaxis, and integrin activation and firm adhesion of monocytes[31, 33-36]. Therefore, the inhibition of CCL2-activated MAPK signaling cascade by APN could lead to anti-inflammatory and atheroprotective effects.

It has been shown that APN expresses its biological functions through associated molecules, such as AdipoR1, AdipoR2 [37], calreticulin[38], and T-cadherin[39]. Particularly, AdipoR1 and AdipoR2, the cell membrane-bound receptors of APN, are involved in insulin sensitivity and antidiabetic effects, whereas calreticulin promotes the removal of apoptotic cells via binding to APN. We have reported several APN-associating molecules, ESL-1 [22], CysC[23], and M2BP[24], and their roles in the pathogenesis of atherosclerosis. ESL-1 is a membrane protein on monocytes involved in the initiation of atherosclerosis through the binding to vascular endothelial E-selectin. Therefore, APN inhibits monocyte adhesion to the vascular endothelium by interfering with the binding between ESL-1 and E-selectin, which could be an anti-atherosclerotic mechanism of APN[22]. Originally, we found that APN decreased the TNF-α-stimulated expression of adhesion molecules on vascular endothelial cells[40]. CysC and M2BP are well-known biomarkers of chronic kidney disease (CKD) and non-alcoholic steatohepatitis (NASH), respectively. The atheroprotective effects of APN on vascular endothelial cells are suppressed by M2BP or CysC through their direct association with APN, which may explain the increased atherogenicity in patients with CKD and NASH. Here, we demonstrated that CCL2 caused THP-1 cells to lose their migration ability after incubation with APN. Taken together, the binding between APN and its associated molecules mutually regulates monocyte adhesion to vascular endothelial cells via these three pathways, chemokine CCL2, monocyte ESL-1, and endothelial E-selectin.

The present study suggests that the reciprocal role of CCL2 and APN against atherosclerosis could be controlled by their direct interaction. Further investigation of the serum APN/CCL2 complex should reveal that it would be a useful biomarker for CVD.

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Disclosure

There are no financial conflicts of interest to disclose.

Contribution Statement

M.F., H.Y., N.Y., and R.O. performed research and clinical data analysis; T.M. performed clinical work and analysis; H.Y. and S.K. designed research, and M.F., H.Y., and S.K. wrote the manuscript.

References

1) Frostegard J, Ulfgren AK, Nyberg P, Hedin U, Swedenborg J, Andersson U and Hansson GK: Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. Atherosclerosis, 1999; 145: 33-43
2) Libby P, Ridker PM and Hansson GK: Progress and challenges in translating the biology of atherosclerosis. Nature, 2011; 473: 317-325
3) Newby AC: Metalloproteinases and vulnerable atherosclerotic plaques. Trends Cardiovasc Med, 2007; 17: 253-258
4) Badimon L, Padro T and Vilahur G: Atherosclerosis, platelets and thrombosis in acute ischaemic heart disease. Eur Heart J Acute Cardiovasc Care, 2012; 1: 60-74
5) Stefanadis C, Antoniou CK, Tsiachris D and Pietri P: Coronary Atherosclerotic Vulnerable Plaque: Current Perspectives. J Am Heart Assoc, 2017; 6:
6) Moore MP, Spencer T, Salter DM, Kearney PP, Shaw TR, Starkey IR, Fitzgerald PJ, Erbel R, Lange A, McDicken NW, Sutherland GR and Fox KA: Characterisation of coronary atherosclerotic morphology by spectral analysis of radiofrequency signal: in vitro intravascular ultrasound study with histological and radiological validation. Heart, 1998; 79: 459-467
7) Nair A, Kuban BD, Tuzcu EM, Schoenhagen P, Nissen SE and Vince DG: Coronary plaque classification with intravascular ultrasound radiofrequency data analysis. Circulation, 2002; 106: 2200-2206
8) Matsuzawa Y, Funahashi T, Kihara S and Shimomura I: Adiponectin and metabolic syndrome. Arterioscler Thromb Vasc Biol, 2004; 24: 29-33
9) Ouchi N, Kihara S, Funahashi T, Matsuzawa Y and Walsh K: Obesity, adiponectin and vascular inflammatory disease. Curr Opin Lipidol, 2003; 14: 561-566
10) Inoue T, Kotooka N, Morooka T, Komoda H, Uchida T, Aso Y, Inukai T, Okuno T and Node K: High molecular weight adiponectin as a predictor of long-term clinical outcome in patients with coronary artery disease. Am J Cardiol, 2007; 100: 569-574
11) Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, Arita Y, Okamoto Y, Shimomura I, Hiraoka H, Nakamura T, Funahashi T, Matsuzawa Y and Osaka CADSGCad: Association of hypoadiponectinemia with coronary artery disease in men. Arterioscler Thromb Vasc Biol, 2003; 23: 85-89
12) Laughlin GA, Barrett-Connor E, May S and Langenberg C: Association of adiponectin with coronary heart disease and mortality: the Rancho Bernardo study. Am J Epidemiol, 2007; 165: 164-174
13) Otsuka F, Sugiyama S, Kojima S, Maruyoshi H, Funahashi T, Matsui K, Sakamoto T, Yoshimura M, Kimura K, Umemura S and Ogawa H: Plasma adiponectin levels are associated with coronary lesion complexity in men with coronary artery disease. J Am Coll Cardiol, 2006; 48: 1155-1162
14) Sattar N, Wannamethee G, Sarwar N, Tchernova J, Cherry L, Wallace AM, Danesh J and Whincup PH: Adiponectin and coronary heart disease: a prospective study and meta-analysis. Circulation, 2006; 114: 623-629
15) Pischon T, Girman CJ, Hotamisligil GS, Rifai N, Hu FB and Rimm EB: Plasma adiponectin levels and risk of myocardial infarction in men. JAMA, 2004; 291: 1730-1737
16) Kobayashi H, Ouchi N, Kihara S, Walsh K, Kumada M, Abe Y, Funahashi T and Matsuzawa Y: Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin. Circ Res, 2004; 94: e27-31
17) Tabas I and Lichtman AH: Monocyte-Macrophages and T Cells in Atherosclerosis. Immunity, 2017; 47: 621-634
18) Weber C, Schober A and Zernecke A: Chemokines: key regulators of mononuclear cell recruitment in atherosclerotic vascular disease. Arterioscler Thromb Vasc Biol, 2004; 24: 1997-2008
19) Zernecke A, Shagdarsuren E and Weber C: Chemokines in atherosclerosis: an update. Arterioscler Thromb Vasc Biol, 2008; 28: 1897-1908
20) Boring L, Gosling J, Cleary M and Charo IF: Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. Nature, 1998; 394: 894-897
21) Gosling J, Slaymaker S, Gu L, Tseng S, Zlot CH, Young SG, Rollins BJ and Charo IF: MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. J Clin Invest, 1999; 103: 773-778
22) Yamamoto H, Kuroda N, Uekita H, Kochi I, Matsumoto A, Niinaga R, Funahashi T, Shimomura I and Kihara S: E-selectin ligand-1 (ESL-1) is a novel adiponectin binding protein on cell adhesion. Biochem Biophys Res Commun, 2016; 470: 425-430
23) Matsumoto A, Yamamoto H, Matsuoka T, Kayama K, Onishi S, Matsuo N and Kihara S: Cystatin C-Adiponectin Complex in Plasma Associates with Coronary Plaque Instability. J Atheroscler Thromb, 2017; 24: 970-979
24) Niinaga R, Yamamoto H, Yoshii M, Uekita H, Yamane N, Kochi I, Matsumoto A, Matsuoka T and Kihara S: Marked elevation of serum M2BP-adiponectin complex in men with coronary artery disease. Atherosclerosis, 2016; 253: 70-74
25) Masaie H, Oritani K, Yokota T, Takahashi I, Shirogane T, Ujiie H, Ichii M, Saitoh N, Maeda T, Tanigawa R, Oka K, Hoshida Y, Tomiyama Y and Kanakura Y: Adiponectin binds to chemokines via the globular head and modulates interactions between chemokines and heparan sulfates. Exp Hematol, 2007; 35: 947-956
26) Sawada T, Shite J, Shinke T, Otake H, Tanino Y, Ogasawara D, Kawamori H, Kato H, Miyoshi N, Yoshino N, Kozuki A and Hirata K: Low plasma adiponectin levels are associated with presence of thin-cap fibroatheroma in men with stable coronary artery disease. Int J Cardiol, 2010; 142: 250-256
27) Otake H, Shite J, Shinke T, Watanabe S, Tanino Y, Ogasawara D, Sawada T, Hirata K and Yokoyama M: Relation between plasma adiponectin, high-sensitivity C-reactive protein, and coronary plaque components in patients with acute coronary syndrome. Am J Cardiol, 2008; 101: 1-7
28) Fuchs S, Lavi I, Tzang O, Bessler H, Brosh D, Bental T, Dvir D, Einav S and Kornowski R: Intracoronary monocyte chemoattractant protein 1 and vascular endothelial growth factor levels are associated with necrotic core, cal-

cium and fibrous tissue atherosclerotic plaque components: an intracoronary ultrasound radiofrequency study. Cardiology, 2012; 123: 125-132
29) Cheng JM, Oemrawsingh RM, Akkerhuis KM, Garcia-Garcia HM, de Boer SP, Battes LC, Buljubasic N, Lenzen MJ, de Jaegere PP, van Geuns RJ, Serruys PW, Kardys I and Boersma E: Circulating chemokines in relation to coronary plaque characteristics on radiofrequency intravascular ultrasound and cardiovascular outcome. Biomarkers, 2014; 19: 611-619
30) Macanas-Pirard P, Quezada T, Navarrete L, Broekhuizen R, Leisewitz A, Nervi B and Ramirez PA: The CCL2/CCR2 Axis Affects Transmigration and Proliferation but Not Resistance to Chemotherapy of Acute Myeloid Leukemia Cells. PLoS One, 2017; 12: e0168888
31) Ashida N, Arai H, Yamasaki M and Kita T: Distinct signaling pathways for MCP-1-dependent integrin activation and chemotaxis. J Biol Chem, 2001; 276: 16555-16560
32) Lopez-Ilasaca M: Signaling from G-protein-coupled receptors to mitogen-activated protein (MAP)-kinase cascades. Biochem Pharmacol, 1998; 56: 269-277
33) Kim HS, Ullevig SL, Zamora D, Lee CF and Asmis R: Redox regulation of MAPK phosphatase 1 controls monocyte migration and macrophage recruitment. Proc Natl Acad Sci U S A, 2012; 109: E2803-2812
34) Ayala JM, Goyal S, Liverton NJ, Claremon DA, O'Keefe SJ and Hanlon WA: Serum-induced monocyte differentiation and monocyte chemotaxis are regulated by the p38 MAP kinase signal transduction pathway. J Leukoc Biol, 2000; 67: 869-875
35) Yen H, Zhang Y, Penfold S and Rollins BJ: MCP-1-mediated chemotaxis requires activation of non-overlapping signal transduction pathways. J Leukoc Biol, 1997; 61: 529-532
36) Cambien B, Pomeranz M, Schmid-Antomarchi H, Millet MA, Breittmayer V, Rossi B and Schmid-Alliana A: Signal transduction pathways involved in soluble fractalkine-induced monocytic cell adhesion. Blood, 2001; 97: 2031-2037
37) Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K and Tobe K: Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. J Clin Invest, 2006; 116: 1784-1792
38) Takemura Y, Ouchi N, Shibata R, Aprahamian T, Kirber MT, Summer RS, Kihara S and Walsh K: Adiponectin modulates inflammatory reactions via calreticulin receptor-dependent clearance of early apoptotic bodies. J Clin Invest, 2007; 117: 375-386
39) Hug C, Wang J, Ahmad NS, Bogan JS, Tsao TS and Lodish HF: T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. Proc Natl Acad Sci U S A, 2004; 101: 10308-10313
40) Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T and Matsuzawa Y: Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. Circulation, 1999; 100: 2473-2476