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Resistin is secreted from macrophages in atheromas and promotes atherosclerosis

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Abstract

Objective: Resistin belongs to a family of cysteine-rich secreted polypeptides that are mainly produced by monocytes/macrophages in humans. Recently, high concentrations of resistin were shown to induce vascular endothelial dysfunction and vascular smooth muscle cell proliferation. We examined if resistin was secreted from macrophages locally in atheromas and if it affected vascular cell function in human.
Methods and results: Immunohistochemical staining of human vessels showed that aortic aneurysms exhibited resistin-positive staining areas along macrophage infiltration, while normal arteries and veins did not. Co-localization of resistin and CD68 (a marker for macrophages) was observed in immunofluorescent double staining of aneurysms. Resistin mRNA was expressed much higher in cultured monocytes/macrophages than in human vascular smooth muscle cells (VSMCs) and human umbilical venous endothelial cells (HUVECs). This suggested that the resistin in aneurysms originates from macrophages within the vessels. To determine the effects of resistin on atherosclerosis, HUVECs and human VSMCs were incubated with resistin (10–100 ng/mL for 4~24 h). In HUVECs, plasminogen activator inhibitor (PAI)-1 release was assayed by ELISA, while the PAI-1 and endothelin (ET)-1 mRNA levels were analyzed by Northern blotting. Both were increased significantly with resistin treatment by factors of 1.3–2.5 ($p<0.05$). Migratory activity of VSMCs measured by scratched wound assay also increased significantly (1.6 times, $p<0.05$). In summary, macrophages infiltrating atherosclerotic aneurysms secrete resistin, and resistin affects endothelial function and VSMC migration.
Conclusions: Resistin secreted from macrophages may contribute to atherogenesis by virtue of its effects on vascular endothelial cells and smooth muscle cells in humans.

Keywords: Resistin; Macrophages; Atherosclerosis; PAI-1; Migration

See also Editorial by J. Hermann (pages 9–12) in this issue.

Abbreviations: AAA, abdominal aortic aneurysm; BMI, body mass index = weight in kilogram / height in square meter; Ct, threshold cycle; DM, diabetes mellitus; ELISA, enzyme-linked immunosorbent assay; ET-1, endothelin type 1; FACS, Fluorescence-activated cell sorting; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HT, hypertension; HUVECs, human umbilical venous endothelial cells; IF, immunofluorescent; IHC, immunohistochemical; mAb, monoclonal antibody; PAI-1, plasminogen activator inhibitor type 1; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; TNF, tumor necrosis factor; VSMCs, vascular smooth muscle cells.

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Resistin belongs to a novel family of cysteine-rich proteins, which have unique patterns of tissue expression [1–3]. Resistin was initially identified in rodents as an adipose tissue-specific secreted protein that is down-regulated by PPAR gamma agonist [3]. Subsequent studies suggested that resistin causes increased hepatic glucose production, and that this leads to insulin resistance [4–6]. Although resistin has been implicated as a link between obesity and insulin resistance in rodents, the role of resistin in humans is unclear, because unlike the situation in rodents, it is expressed at higher levels in monocytes and macrophages than in adipocytes in humans [7–10]. The abundant expression of resistin in monocytes/macrophages in humans suggests that it might have another pathophysiologic role. Indeed, several have reported that resistin induces vascular endothelial dysfunction and vascular smooth muscle cell proliferation [11–13]. However, the concentrations of resistin that showed definitive effects in these studies were much higher than physiologic concentrations (<20 ng/mL) [14–20].

On considering the potential role of resistin in atherogenesis, we hypothesized that resistin secretion from macrophages in atherosclerotic arteries could affect vascular cell function and potentially contribute to atherosclerosis. Based on this hypothesis, we examined whether macrophages that have infiltrated atherosclerotic arteries secrete resistin locally. Additional evidence for the relationships between resistin and endothelial dysfunction or VSMC migration was obtained from in vitro experiments.

1. Materials and methods

This study conformed with the principles outlined in the Declaration of Helsinki. The institutional review board of Seoul National University Hospital approved the study protocol, and informed consent was obtained from all subjects.

1.1. Preparation of human samples

To isolate circulating monocytes, whole blood was extracted from healthy volunteers aged <30 years. To isolate human VSMCs, gastroepiploic arteries were extracted from patients undergoing gastric surgery without clinical atherosclerosis or the risk of developing this disease.

To compare resistin expression in atherosclerotic arteries and normal vessels, three categories of patients were selected. First, four patients scheduled for abdominal aortic aneurysm resection: their clinical details are presented in Table 1. Second, three patients (<40 years old) scheduled for varicose vein stripping, with no risk factors of atherosclerosis, were assigned as normal controls. Third, a single 30-yr-old healthy donor scheduled for renal transplantation was assigned as another normal control. In this case, only a small sample of a renal artery was available for immunohistochemical staining.

1.2. Cell culture and treatment: circulating monocytes, HUVECs, human VSMCs

Circulating monocytes were isolated from peripheral blood mononuclear cells by adherence to uncoated plates. The peripheral blood mononuclear cells were freshly prepared by density gradient centrifugation using Ficoll-Paque Plus™ (Amersham Biosciences, Piscataway, NJ). Adherent cells were characterized as monocytes by FACS using anti-CD14 mAb. The cells were then incubated for 36 h with RPMI1640 medium (GibcoBRL, Long Island, NY) supplemented with 10% FBS, 20 μM 2-mercaptoethanol and 10 mM HEPES (all reagents were obtained from Sigma-Aldrich, Saint Louis, Missouri).

HUVECs at passage 8–12 were cultured in EGM-2 medium (both obtained from Cambrex BioScience, New Jersey) until 70% confluent. Human recombinant resistin (Phoenix Pharmaceuticals, Belmont, CA) was added to medium at 10, 50 or 100 ng/mL and incubated for 4∼24 h. As a positive control, HUVECs were also incubated with 10 ng/mL of human recombinant tumor necrosis factor (TNF)-α (R&D Systems, Minneapolis) for 12 h.

Human VSMCs were isolated from human gastroepiploic arteries by using an enzymatic dispersion method [21] and were characterized by immunocytochemically using mono-

Table 1
Clinical data of subjects with abdominal aortic aneurysms

Sample number	#1	#2	#3	#4
Age (years)	70	69	66	54
Sex	Male	Male	Male	Male
BMI (kg/m^2)	24.8	24.4	20.8	26.1
Indication for surgery	Impending rupture and pain	Increase of size	Increase of size	Increase of size
DM/HT/dyslipidemia	−/+/−	−/+/+	−/−/−	−/+/−
Smoking	+	−	−	Ex-smoker (4 years)
Pathologic finding	Most part is thrombus with atheromatous plaque and inflammation	Most part is thrombus with atheromatous plaque and inflammation	Atheromatous vessel with thrombus and inflammation	Atheromatous vessel with thrombus and inflammation
Dissection	−	−	−	−
Penetrating ulcer	−	−	−	+
Intramural hemorrhage	+	−	−	+

clonal anti-α-smooth muscle actin antibodies (Sigma-Aldrich Co., Saint Louis, Missouri) and by morphology. Cells at passage 6–10 were cultured in DMEM (GibcoBRL, Long Island, NY) supplemented with 10% FBS until 80% confluent. To measure cell migration, cells were incubated in serum-free medium for 24 h, and then human recombinant resistin (Phoenix Pharmaceuticals, Belmont, CA) was added at 10, 50, or 100 ng/mL and incubated for 24 h. As a positive control, FBS was added and the cells were incubated for 24 h.

1.3. Immunohistochemical staining and immunofluorescent double staining: human vessels

Immunohistochemical (IHC) studies and immunofluorescent (IF) double stainings were performed on formalin-fixed, paraffin-embedded tissues. These tissues were: human abdominal aortic aneurysms ($n=4$), varicose veins ($n=3$) and normal renal artery ($n=1$). Aneurysm samples were prepared from transition zones and centers. Tissue blocks were sectioned at 4 μm, attached to silane-coated slides, deparaffinized in xylene, and rehydrated in graded alcohol.

For IHC staining, deparaffinized sections were boiled at 125 °C for 1 min in Target Retrieval solution (pH 9.0 Tris/EDTA buffer), and treated with 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were then incubated with primary antibodies, namely, monoclonal anti-human resistin (R&D Systems, Minneapolis, clone 184335), and anti-human CD68 (DakoCytomation, Glostrup, Denmark, clone KP1), the latter of which is a marker for monocytes/macrophages [22]. EnVision+™ mouse peroxidase and 3,3′-diaminobenzidine tetrahydrochloride (DAB) were used as secondary antibody and chromogen, respectively (both from DakoCytomation). Mayer's hematoxylin was used for counterstaining. As a negative control, the primary antibody was replaced with Tris Buffered Saline–Tween 20.

For IF double staining, deparaffinized sections were fixed in 100% cold methanol and non-specific proteins were blocked with 1% bovine serum albumin. The sections were then incubated with antibodies as follows: as 1st primary antibodies, anti-CD68 (clone KP1), anti-CD31 (clone JC70A), or anti-smooth muscle actin (clone 1A4) (all from DakoCytomation, Glostrup, Denmark); 1st secondary antibody (Alexa Fluor 488, Molecular Probe, Eugene, OR); 2nd primary antibody, anti-human polyclonal resistin (KOMED, Seoul, Korea); and 2nd secondary antibody (Alexa Fluor 594, Molecular Probe). Finally, DAPI staining was performed to identify nuclei.

1.4. Real-time PCR of resistin mRNA in human vessels, cultured vascular cells, and cultured monocytes

Total RNA was extracted from the centers of abdominal aortic aneurysms ($n=4$), varicose veins ($n=3$), cultured HUVECs, VSMCs and from monocytes using a Trizol kit (Invitrogen Corp., Carlsbad, California). Reverse transcription was performed using 1.5 μg of total RNA in 20 μL using a Reverse Transcription Kit (Invitrogen). For real-time PCR assays, a master mix of the following components was prepared, at the indicated final concentrations: 2.5 μL each primer (9 μM), 2.5 μL probe (2.5 μM), 2.5 μL water, and 12.5 μL TaqMan PCR 2× master mixture (Applied Biosystems, Lincoln, CA). The PCR primers and probes used are listed in Table 2. Five microliters of reverse transcription reaction mixture was added as a PCR template. Relative quantitative real-time PCR was performed using the above reagents using an ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems, Lincoln, CA). The following procedure was used. After initial activation of uracyl-*N*-glycosylase at 50 °C for 2 min, AmpliTaq Gold was activated at 95 °C for 10 min. PCR consisted of 45 amplification cycles (denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and extension at 60 °C for 1 min). During PCR amplification, the amplified product amount was monitored by continuous measurement of fluorescence. The expression of resistin gene was normalized versus GAPDH (VIC/MGB probe, primer limited) as follows; the cycle number at which the transcript of the resistin gene was detectable (threshold cycle, Ct) was normalized against Ct of GAPDH, which is referred to as deltaCt. The expression of resistin relative to a reference was expressed as $2^{-\mathrm{deltadeltaCt}}$, where deltadeltaCt refers to the difference in the values of deltaCt between the test groups and the reference. Intra-assay coefficient of variation (CV) of Ct was under 8.6% for resistin and under 1.2% for GAPDH.

1.5. Northern blot and protein assay: HUVECs

Total RNA was purified from HUVECs using a Trizol kit (Invitrogen), electrophoresed through an agarose gel containing formaldehyde, and then transferred to nitrocellulose membranes. Membranes were hybridized with the [α-^{32}P]-labeled cDNAs of human PAI-1 (plasminogen activator inhibitor type 1) or ET-1 (endothelin type 1). The primers used for probe synthesis are listed in Table 2. Hybridization with human GAPDH cDNA was used as an internal control. Quantitation of relative signal intensities was performed

Table 2
Primers and probes used in RT-PCR

Target mRNA		Sequence
Resistin	Forward	TGTGCCGGATTTGGTTAGC
	Reverse	CTCTCATTGATGGCTTCTTCCA
	Probe	CCTGCAGGATGAAAGCTCTCTGTCTCCTC5′ Fluorescein/3′TAMRA
PAI-1	Forward	ACAACTCCCTTAAGGTCTCC
	Reverse	ATTACATCCATCTTTGTGCC
ET-1	Forward	CAGAAACAGCAGTCTTAGGC
	Reverse	GTTTCTCATGGTCTCCGAC

with a densitometer (TINA image analyzing software, Raytest, Straubenhardt, Germany).

PAI-1 protein release from HUVECs was determined in culture medium using an ELISA kit (Biopool, Umea, Sweden). Intra-assay coefficients of variance were <9.9%.

1.6. Migration assay: human VSMCs

VSMC migration was detected using a scratched wound assay. After culturing cells to 80% confluency on 6-well plates previously labeled with a traced line, the cells were starved in serum-free medium for 24 h. In vitro scratches were created by scraping cell monolayers as previously described [23,24]. The remaining cells were washed with medium and incubated with resistin for 24 h. VSMC migrations into scratches were quantified using a digital microscope image analysis system (DMC2, Polaroid Co., Waltham, MA).

2. Results

2.1. Immunohistochemical staining of human vessels: resistin and CD68

Paraffin-embedded vessel sections were stained with anti-resistin antibody (Fig. 1). Two portions of arterial lesions were prepared, i.e., aortic aneurysm transition zones with intact endothelium, smooth muscle cells and some infiltrating inflammatory cells (Fig. 1: C, G, K), and aortic aneurysm centers with thick atheromatous plaque and numerous inflammatory cells but few normal vascular components (Fig. 1: D, H, L). Resistin-positive areas were seen only in atherosclerotic vessels (Fig. 1: C, D: dark region beneath the endothelial lining), especially in aneurysm centers. The resistin-positive areas are distributed along inflammatory cells. These inflammatory cells were also immunoreactive for CD68, a marker for monocytes/macrophages (Fig. 1: G, H). No stained areas were observed by either of the two antibodies in normal blood vessels (Fig. 1: A, B, E, F). Staining without primary Ab was performed as a negative control, and this showed no staining (Fig. 1: I, J, K, L).

2.2. Immunofluorescent double staining of human vessels for resistin, CD68, CD31 and smooth muscle actin

Paraffin-embedded aneurysm sections were immunostained with anti-CD68, anti-smooth muscle actin, or anti-CD31 to identify macrophages, smooth muscle cells, and endothelial cells, respectively (Fig. 2). Green fluorescent areas indicate marked proteins (A, D, G). The 2nd antibody used was anti-resistin and protein expressions were observed as red fluorescent areas (B, E, H). The blue dots in C, F and I indicate nuclei and confirm the presence of cellular components. A, B and C of Fig. 2 show the co-localization of CD68-positive and resistin-stained areas, as in Fig. 1. D, E and F show resistin-positive areas, which constitute portions of smooth muscle cell layers. Fig. 2G, which represents endothelial lining, has no stained area, which means that the endothelium has been denuded. A, B

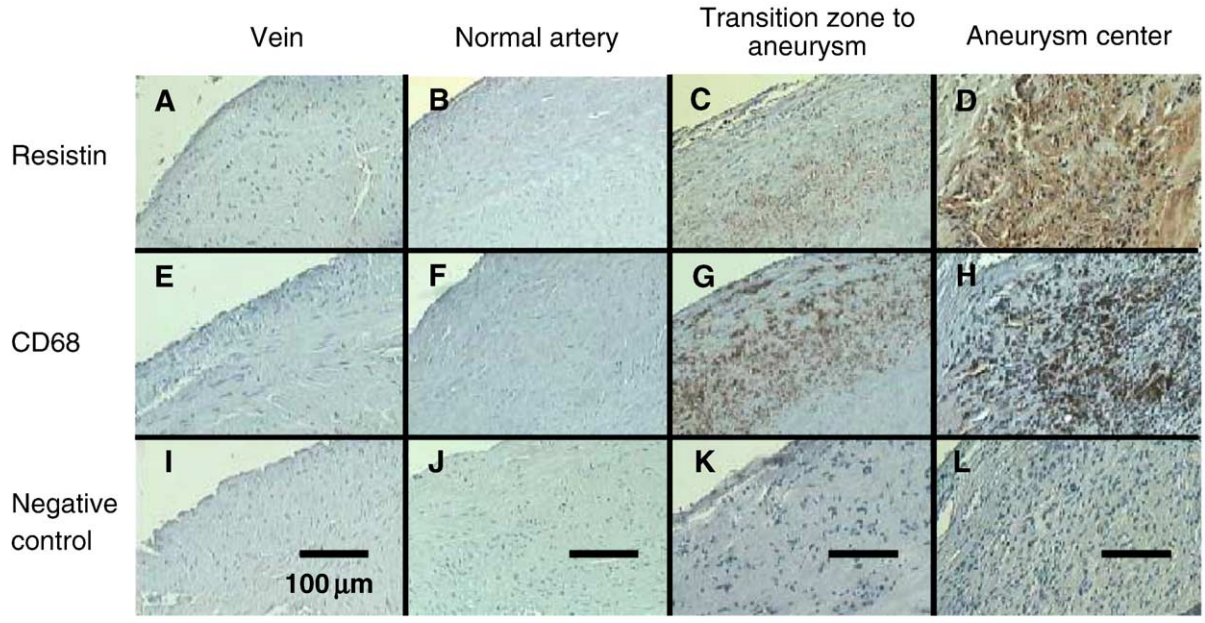

Fig. 1. Immunohistochemical staining of aneurysms with antibodies to resistin and CD68. The various types of human vessels were stained with antibodies against resistin or CD68, the latter of which is a marker of monocytes/macrophages. Mayer's hematoxylin was used for counterstaining. The left upper region of each picture indicates the endothelial lining. Resistin-positive areas are seen in atherosclerotic lesions as dark staining beneath the endothelial lining (C and D). Resistin-positive areas are distributed along inflammatory cells and stained positively for CD68 (G, H). No immunoreactive area was observed for either of the two antibodies in normal vessels (A, B, E, F). Staining without primary Abs was performed as a negative control (I, J, K, L).

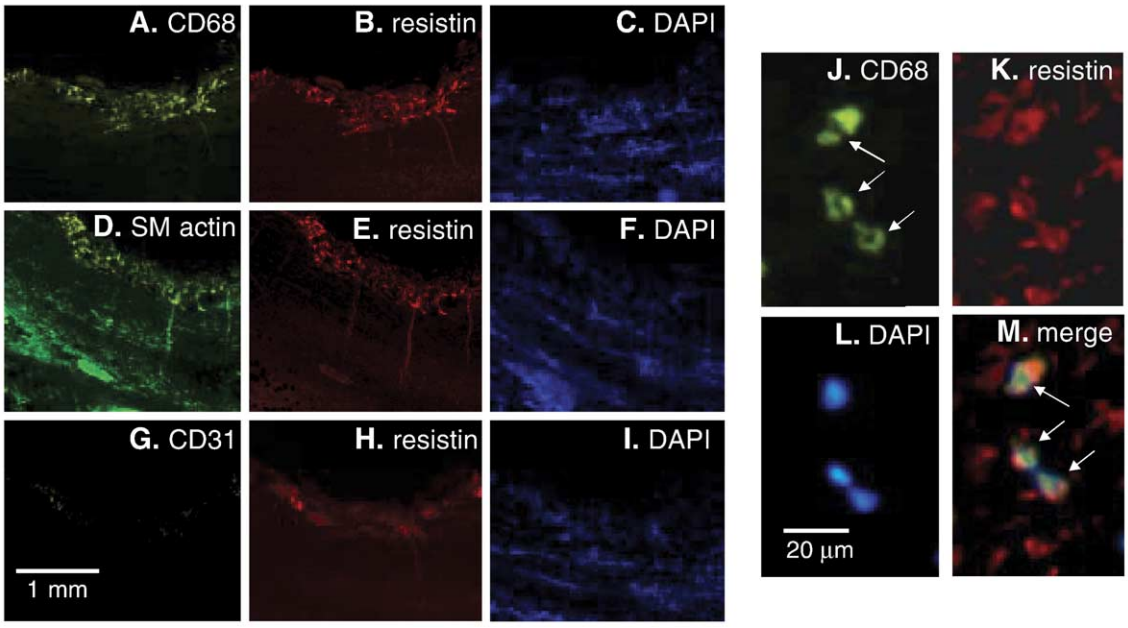

Fig. 2. Immunofluorescent double staining of aneurysms with antibodies to resistin and vascular cell markers. Green indicates vascular cell proteins, red resistin, and blue DAPI-stained cellular nuclei. The upper region in each picture indicates endothelial lining. Resistin protein was found to co-localize with CD68 (A, B). Smooth muscle (SM) actin was positive throughout the vessel thickness (D), whereas CD31 was not detected (G). The distribution of resistin (E, H) was not concordant with either D or G. A, B and C were magnified (J~L) and this showed that resistin was present both intracellularly and extracellularly. The merged picture (M) shows yellow rings composed of red resistin-positive areas and green transmembrane CD68-positive areas (arrows).

and C of Fig. 2 were magnified to assess the intracellular and extracellular locations of resistin (J, K, L). As CD68 is a transmembrane protein, the green-stained areas in figure J are ring-shaped (arrows). Resistin was found to be present in both intracellular and extracellular areas, which was confirmed by merging figures J and K. In the merged picture (figure M), CD68-positive areas and resistin-positive areas formed yellow rings with blue DAPI-stained cores (arrows).

2.3. Resistin mRNA expression from vessels and cultured cells

Real-time quantitative PCR analysis of resistin in the center of abdominal aortic aneurysms with atheromatous plaque and calcification ($n=4$) and in varicose veins ($n=3$) revealed resistin overexpression in the former (Fig. 3A). Resistin mRNA was scarcely expressed in cultured HUVECs or VSMCs stimulated with TNF-α, but was

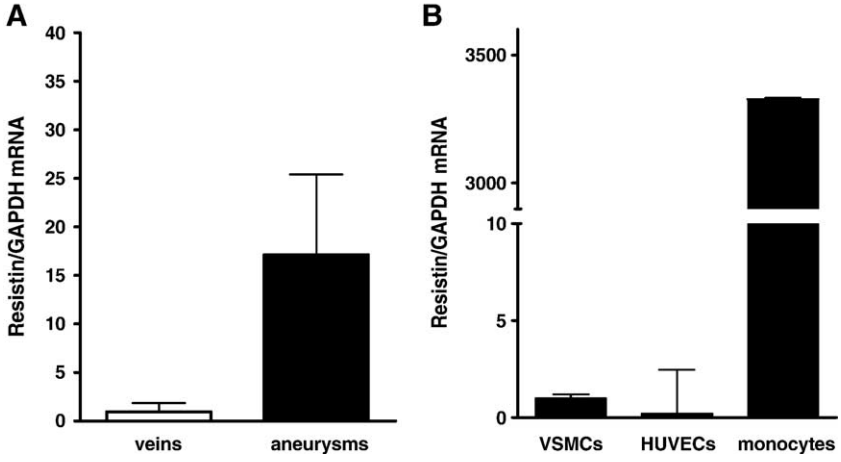

Fig. 3. Resistin mRNA expression measured by real-time PCR. (A) Resistin mRNA expression in aortic aneurysms. Total RNA was purified from aneurysm centers ($n=4$) and veins ($n=3$). Real-time quantitative PCR revealed the overexpression of resistin in aneurysm centers. The y axis represents resistin expression normalized against GAPDH expression. The data illustrated on the graph represent means ± SEM of $2^{-\text{deltadeltaCt}}$ of the samples. (B) Resistin mRNA expression in vascular cells. Isolated human VSMCs and commercial HUVECs were incubated with 10 ng/mL of TNF-α for 12 h. Human monocytes were isolated and incubated for 36 h, and total RNA was purified and resistin mRNA levels were measured by real-time RT-PCR, normalized with respect to the GAPDH mRNA levels. The data illustrated on the graph represent means ± SEM of $2^{-\text{deltadeltaCt}}$ of three different experiments.

highly expressed in the cultured monocytes (Fig. 3B). These results suggest that resistin expressed in aneurysms originates from infiltrating macrophages, and not from normal vascular cells.

2.4. PAI-1 protein assay and the mRNA expression of PAI-1/ET-1 from HUVECs

We evaluated the effects of human recombinant resistin (10 to 100 ng/mL) on PAI-1 production by HUVECs. Incubation of HUVECs with resistin for 24 h increased levels of PAI-1 secretion ($p<0.05$, Fig. 4A). This effect was compared with that induced by TNF-α treatment (10 ng/mL for 12 h) which showed a 1.6-fold increase in PAI-1 secretion ($p<0.05$). Increased PAI-1 secretion was accompanied by increased levels of the PAI-1 transcript. Incubation of HUVECs with resistin (50 or 100 ng/mL) resulted in a 2.5-fold increase in PAI-1 mRNA expression ($p<0.05$, Fig. 4B upper panel). The time-dependent effect of resistin on PAI-1 mRNA levels was also examined (100 ng/mL resistin for 4∼24 h) and was found to be increased from 8 h ($p<0.05$, Fig. 4B lower panel).

In addition, we evaluated the effects of resistin on the expression of ET-1. ET-1 mRNA levels showed significant dose- and time-dependent increases after resistin treatment (10∼100 ng/mL, 4∼24 h), and a 1.5-fold increase was observed after treating with 100 ng/mL of resistin for 4 h ($p<0.05$, Fig. 5).

2.5. Measurement of VSMC migration

Treatment with resistin increased the migration rate of VSMCs in a dose-dependent manner. The maximal change (1.6-fold) was observed after treatment with 100 ng/mL for 24 h ($p<0.05$, Fig. 6).

3. Discussion

Resistin was initially suggested to be a link between obesity and insulin resistance in rodents [2,3], but evidence to confirm this in humans is not sufficient. Instead, resistin expression was found to be abundant in monocytes/macrophages [7–9], which play an important role in atheroscle-

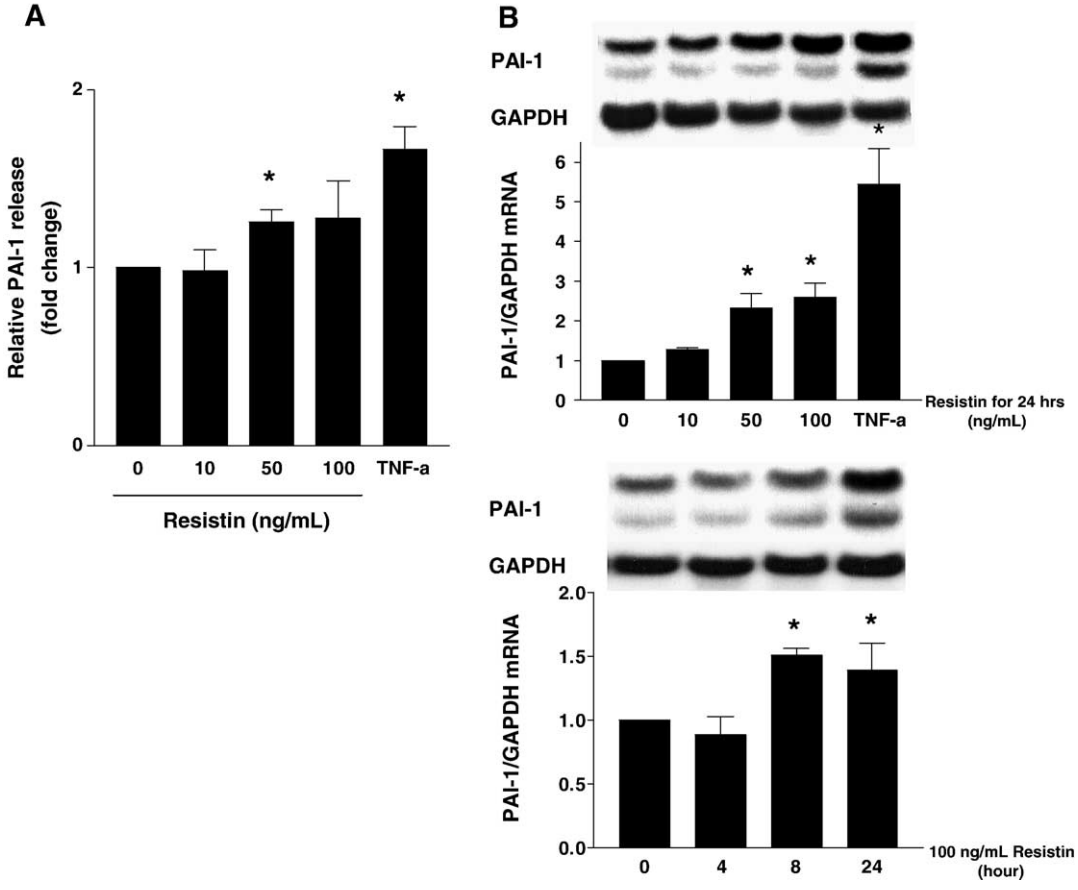

Fig. 4. Induction of PAI-1 in HUVECs after resistin treatment. HUVECs were incubated with 10∼100 ng/mL of resistin for 4∼24 h or with 10 ng/mL of TNF-α for 12 h as a positive control. (A) PAI-1 protein levels in culture media were measured by ELISA and are presented as fold changes versus the negative control. (B) PAI-1 mRNA expression was analyzed by Northern blotting, and normalized against GAPDH mRNA. Results are presented as fold changes versus the negative control. The data illustrated on the graph represent the means±SEM of three different experiments. *$p<0.05$ vs. without resistin treatment.

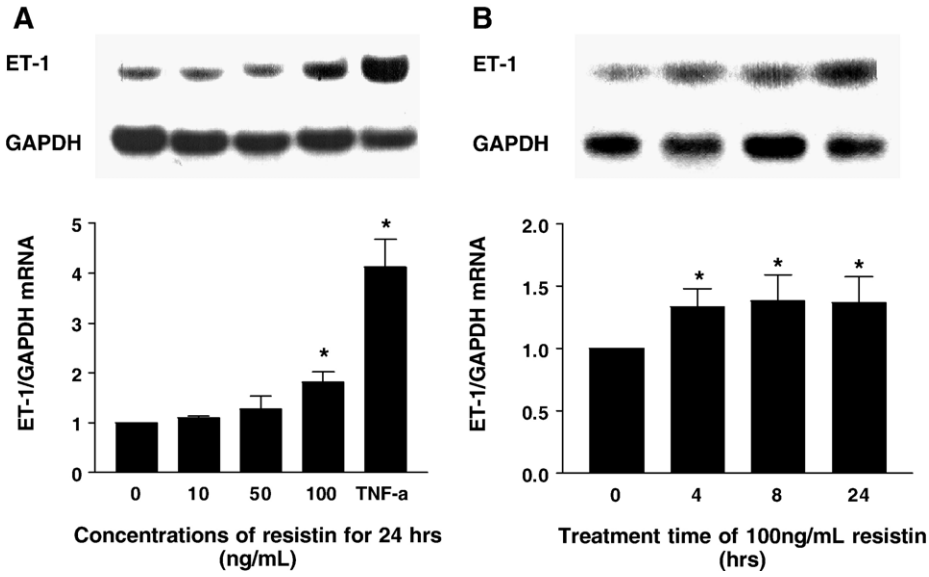

Fig. 5. Induction of ET-1 in HUVECs after resistin treatment. HUVECs were incubated with 10~100 ng/mL of resistin for 4~24 h or with 10 ng/mL of TNF-α for 12 h as a positive control. ET-1 mRNA levels were analyzed by Northern blotting and normalized against GAPDH mRNA, and are presented as fold changes versus the negative control. The data illustrated on the graph represent the means±SEM of three different experiments. *$p<0.05$ vs. without resistin treatment.

rosis [25]. These cells infiltrate arteries and initiate or promote atherogenesis by secreting various pro-inflammatory cytokines [26].

In this study, we found that both resistin protein and its transcript are present in atherosclerotic aneurysms and that resistin protein stains positively around infiltrating macrophages (Figs. 1–3). As shown in Fig. 2, resistin protein was found to co-localize with CD68-positive inflammatory cells, but not with endothelial cells or smooth muscle cells. A higher magnification (Fig. 2: J, K, L, M) revealed that resistin was present both intracellularly and extracellularly, which concurred with the fact that resistin is a secretory protein. To date no report has been issued on resistin-binding proteins or resistin receptors. Resistin expression was found to be much higher in atherosclerotic aneurysms than in normal veins at both the transcript and protein level. Moreover, resistin expression at the cellular level revealed that macrophages were the source of resistin in aneurysms. Cultured monocytes/macrophages expressed resistin mRNA at high levels, but HUVECs and human VSMCs did not (Fig. 3B). From these findings, we conclude that resistin is secreted by macrophages that have infiltrated arteries.

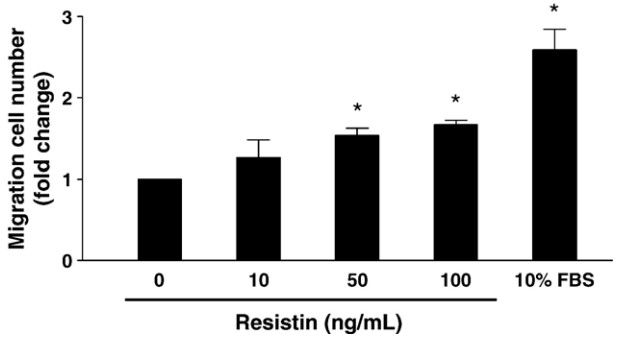

Fig. 6. Induction of VSMC migration by resistin. VSMCs in serum-free media were incubated with 10~100 ng/mL resistin for 24 h or with 10% FBS for 24 h as a positive control. Cell migration was detected by scratched wound assays and results are presented as fold changes versus the negative control. The data illustrated on the graph represent means±SEM of three different experiments. *$p<0.05$ vs. without resistin treatment.

Although abdominal aortic aneurysms (AAAs) are strongly associated with atherosclerosis, clear causal relationship has not been established. The primary pathophysiologic abnormality of AAA is a chronic transmural inflammation associated with the monocytes/macrophage lineage and the destruction of the media [27]. Thus our findings in AAA might not be representative of atherosclerosis, although we observed resistin expression in both the transition zone and in the center of AAAs. Further studies on atheroma from other vascular beds with different stages and normal arteries (as a control) will be helpful to identify a temporal and potentially pathophysiological relationship between resistin expression in the arterial wall and atherogenesis.

Atherosclerosis is characterized by endothelial dysfunction and VSMC proliferation/migration [26]. The localized modulation of the vascular endothelium to a non-adaptive functional state could be termed “endothelial dysfunction.” The term endothelial dysfunction has been used to refer to several pathological conditions, which include altered anticoagulant and anti-inflammatory properties of the endothelium, the aberrant modulation of vascular growth, and the dysregulation of vascular remodeling [28]. Resistin was found to induce endothelial dysfunction in vitro, that is, it increased the expressions or releases of ET-1, vascular cell adhesion molecule-l, intercellular adhesion molecule-1, and

pentraxin-3 and decreased the expression of TNF receptor-associated factor-3 [11,12]. PAI-1 is a prothrombotic molecule which has an important role in endothelial dysfunction and atherosclerosis [29], but the effect of resistin on PAI-1 is not known. In the present study, we found that PAI-1 mRNA and protein were upregulated by resistin (Fig. 4), as was ET-1 mRNA (Fig. 5). Increase of ET-1 expression by resistin has been observed by Verma et al. [11], and we reproduced it. Calabro et al. recently reported that resistin promotes VSMC proliferation, which might occur through both ERK 1/2 and Akt signaling pathways [13]. The present study shows that resistin enhances VSMC migration, which is a known component of atheromatous plaque synthesis.

All of the above-mentioned effects of resistin on atherosclerosis were clearly observed at concentrations >50 ng/mL. Human plasma resistin concentrations examined to date have generally fallen in the range 5∼20 ng/mL [14–20], and this wide variation appears to be the result of ELISA non-standardization. Only two groups have reported resistin levels exceeding 30 ng/mL, but in these studies differences between resistin concentrations in normal subjects and diabetic patients were not significant [30,31], which contradicts other reports [19,32–34]. Consequently, the physiologic plasma levels of resistin in humans probably do not exceed 20 ng/mL. Considering the resistin concentrations needed to cause endothelial dysfunction and VSMC growth in vitro, locally secreted resistin in atheroma, rather than circulating resistin, would be expected to have a much greater influence on these processes. In the light of our aneurysm IHC staining results, the local concentration of resistin in it appears to be reasonably high. Kawanami et al. reported that resistin levels as high as 1000 ng/mL were not toxic to cultured endothelial cells and induced more adhesion molecule expression than lower "physiologic plasma concentrations" [12]. However, the effect of the circulating form of resistin cannot be entirely ruled out, because a recent report showed a 4- to 5-fold increase in circulating resistin levels in response to cytokine treatment [35]. In this study, endotoxin treatment and resultant inflammatory cytokine secretion seemed to regulate the resistin expressions of monocytes/macrophages via the NF-kappaB pathway because blocking this pathway attenuated resistin expression [35].

There are well established links between metabolic syndrome and cardiovascular disease, such as hypertension, obesity, hyperglycemia, dyslipidemia, and hyperinsulinemia. Recent studies also indicate a reduced adiponectin level and an increased C-reactive protein level as links between them [36–41]. At the moment, there is little to suggest a direct relationship between resistin and obesity, insulin resistance or metabolic syndrome in humans. Our results supported a role of resistin in atherosclerosis, as resistin was found to be secreted by monocytes/macrophages that infiltrated arterial walls, and to induce endothelial dysfunction and VSMC migration in vitro. Thus, resistin might be a monocytes/macrophage factor associated with atherosclerosis. Since resistin is expressed mainly in inflammatory cells [8,9], regulated by and regulating inflammatory cytokines [35,42], and increased in serum in subclinical inflammatory conditions [19,32–34,43–45], it may be a link between inflammation and atherosclerosis. However, we cannot exclude the possibility that resistin might also be a contributory factor to insulin resistance or metabolic syndrome. Recent evidence suggests that subacute hepatic inflammation through NF-kappaB activation and downstream cytokine production cause insulin resistance both locally in the liver and systemically [46].

The association between resistin and atherosclerosis remains controversial. Diez et al. were unable to show any difference in the serum concentrations of resistin between end-stage renal disease patients with or without vascular disease [47]. On the other hand, in a prediabetic/diabetic patient population Shetty et al. found a significant positive correlation between resistin and CRP serum concentrations, and a significant negative correlation between resistin and HDL serum concentrations or nitroglycerin-induced dilatation, although the latter was not confirmed by bivariate and multivariate analyses [44]. Recently, Reilly et al. were able to show that coronary artery calcium score increased with increasing serum resistin concentration in men. In this SIRCA substudy, plasma levels of resistin, in contrast to CRP, were of incremental value in the association between coronary artery disease and metabolic syndrome [45]. As we discussed earlier, resistin is more likely to affect atherosclerosis as a local inflammatory factor, although its systemic effects cannot be ruled out. It remains to be elucidated whether hyperresistinemia is only a marker of inflammation or whether it has systemic effects on atherosclerosis.

In summary, we observed that macrophages infiltrated atherosclerotic aneurysms and secreted resistin, and that resistin induced the productions of PAI-1 and ET-1 by endothelial cells and the migration of VSMCs. We conclude that resistin from macrophages may contribute to atherogenesis by virtue of its effects on vascular endothelial and smooth muscle cells.

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