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Original

Visceral Fat Accumulation is Associated with Oxidative Stress and Increased Matrix Metalloproteinase-9 Expression in Atherogenic Factor-overlapped Model Rats

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Abstract : Visceral fat accumulation in lifestyle-related diseases increases the risk of atherosclerosis. Matrix metalloproteinases (MMPs) play an important role in the progression of atherosclerosis. We examined atherogenic factor-overlapped model rats to clarify the relationships among visceral fat, oxidative stress, and MMPs. We used four groups of male, 11-month-old, spontaneously hypertensive hyperlipidemic rats (SHHRs) or Sprague-Dawley (SD) rats. Animals were fed either a diet of high fat and 30% sucrose solution (HFDS) or a normal diet (ND) ad libitum for 6 months. The visceral fat weight increased by approximately three fold in SHHR-HFDS compared to SHHR-ND. The oxidative stress marker in plasma and MMP-9 mRNA expression in white blood cells increased in SHHR-HFDS compared to the other groups. A correlation was determined between oxidative stress and visceral fat or MMP-9 mRNA in all rats. Lipid deposition and immunostaining of CD68 and MMP-9 were observed mainly in the intima of aorta in SHHR-HFDS, while tissue inhibitor of metalloproteinase-1 mRNA expression decreased in both SHHR groups. The findings suggested that increased oxidative stress due to the visceral fat accumulation induced MMP-9 expression and macrophage accumulation in the intima of aorta in lifestyle-related disease model rats.

Key words : visceral fat, oxidative stress, matrix metalloproteinase-9, macrophage

Introduction

Hypertension, hyperlipidemia, and impaired glucose tolerance are symptoms of lifestyle-related diseases that may lead to cardiovascular and cerebrovascular diseases[1,2)]. Atherosclerosis is the causative factor in most such diseases[3)].

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There are few rat models for atherosclerosis, since rats are an atherosclerosis-resistant species. Therefore, we developed a spontaneously hypertensive hyperlipidemic rat (SHHR) model to study atherogenic factors in the presence of combined hypertension and hyperlipidemia. SHHRs have persistently high levels of systolic blood pressure (SBP) above 150 mmHg and a plasma total cholesterol concentration above 150 mg / dL. In addition, vascular intimal lesions and lipid deposits were observed under endothelial cells in the aorta of SHHRs, but not spontaneously hyperlipidemic rats (HLRs) and controls[4, 5]. Therefore, SHHRs provide a stable model of early vascular degeneration[6]. Moreover, invasive changes occur in the subendothelium of SHHR when nitric oxide (NO) production is inactivated followed by a high fat diet and sucrose water treatment (SHHR-HDFS)[7]. Furthermore, a previous study found visceral fat accumulation and increased oxidative stress in SHHR-HFDS[8].

Matrix metalloproteinases (MMPs) are zinc ion-dependent enzymes that help to regulate the extracellular matrix, cellular migration, and tissue remodeling[9, 10]. MMPs are associated with cardiovascular disease and atherosclerosis; in particular, MMP-2 and MMP-9 play important roles in the progression of atherosclerosis[11], and MMP-9 activity is associated with oxidative stress in acute coronary syndrome[12]. Our previous studies showed that plasma MMP-9 activity is significantly higher and accompanied by aortic lipid deposition in SHHR compared to Sprague-Dawley (SD) control rats[5]. MMP-9's role in the pathophysiology of atherosclerosis and plaque rupture[13] is tightly regulated by tissue inhibitors of metalloproteinases (TIMPs)[14]. In particular, MMP-9 is associated with TIMP-1, a multifunctional protein with the capacity to modulate matrix turnover[15].

$CD8^+$ T cells and macrophages have been linked to considerable amplification of the inflammatory state in adipose tissue, through the secretion of cytokines and chemokines[16, 17]. Moreover, systemic oxidative stress is associated with visceral fat accumulation and the metabolic syndrome[18, 19]. We have previously used the diacron reactive oxidative metabolites (d-ROMs) test as an indicator of oxidative stress[20]. In this study, we examined SHHRs as an atherogenic factor-overlapped animal model to clarify the relationship among visceral fat, oxidative stress, and MMPs[8].

Materials and Methods

Animals and samples

Four-month-old male SHHRs and SD rats were divided into two groups: a control group fed a regular diet (ND, CE2; CLEA Japan, Tokyo) and an HFDS-fed group. The regular diet comprised 8.9% water, 25.4% protein, 4.4% fat, 4.1% fiber, 6.9% carbohydrate, and 50.3% nitrogen-free extracts, containing 342.2 kcal / 100 g. The high-fat diet (HFD) consisted of 8.2% water, 23.4% protein, 11.0% fat, 3.8% fiber, 6.3% carbohydrate, and 46.3% nitrogen-free extracts, containing 378.0 kcal / 100 g. SD-ND, SD-HFDS, SHHR-ND and SHHR-HFDS groups contained 5, 7, 5 and 12 rats, respectively. Until the age of 4 months,

the regular diet was available to all groups ad libitum. Thereafter, the two HFDS groups received NG-nitro-L-arginine methyl ester (L-NAME ; Nacalai Tesque Co., Kyoto, Japan) in the drinking water (100 mg / L) for 1 month, and then the high-fat diet with 30% sucrose solution ad libitum for 6 months, thus from 5 to 11 months of age. The present study used SHHR in which the systolic blood pressure was over 150 mmHg, as determined by the tailcuff method (PS-100 ; Riken Kaihatsu, Tokyo). The rats were housed in a semi-barrier system under controlled room temperature (23 ± 1°C), humidity (55 ± 5%), and lighting (lights on from 6 a.m. to 6 p.m.). All studies were conducted according to the "Guiding Principles for the Care and Use of Laboratory Animals" of Showa University[21].

Preparation and biochemical determination of plasma samples

Blood samples were taken from the inferior vena cava under pentobarbital anesthesia (35 mg / kg, intraperitoneal administration) and mixed with 3.2% sodium citrate solution in a volume ratio of 9 : 1. After 15 min of centrifugation at 3,000 rpm, the supernatant, as citrated plasma, was used for analysis. First, the epididymal fat (EpidF) and visceral fat (VisF) were isolated, and then the visceral fat weight / body weight ratio (VisF / BW) was calculated.

Plasma levels of total cholesterol (TC) and total triglycerides (TG) were determined with commercially available kits (Cholesterol E-test and Triglyceride E-test, respectively ; Wako Pure Chemicals)[8]. Oxidative stress was measured by a d-ROMs test of rat plasma (Free Radical Elective Evaluator ; Wismerll Co. Ltd., Tokyo, Japan)[20, 22]. A 20 μL plasma sample and 1 mL of buffer solution were gently mixed in a cuvette, before adding 10 μL of the chromogenic substrate. After mixing, the cuvette was immediately incubated in the thermostatic block of the analyzer for 5 min at 37°C, and then absorbance at 505 nm was recorded. Measurements are expressed as Carr Units, with 1 Carr corresponding to 0.8 mg / 1 H_2O_2[23].

Preparation of total RNA

Blood samples (300 μL) and Catrimox-14 Solution (3,000 μL) were mixed (Takara Bio, Shiga, Japan), before total RNA was isolated using the Takara FastPure RNA kit and quantified by optical density (260 nm). Each RNA sample was diluted with RNase-free water and mRNA concentrations were equalized. Total RNA was reverse transcribed using the ExScript™ RT reagent kit (Takara).

Quantitative Real-Time PCR

Q-PCR was used to quantify the mRNA levels of MMP-8, MMP-9, and TIMP-1 in rat white blood cells (WBCs). The cDNAs were amplified using primers designed by ProbeFinder software (Roche Diagnostics K.K., Tokyo, Japan). Table 1 details the primers used, Roche Universal ProbeLibrary Probe numbers, and accession numbers. Amplification was performed with a LightCycler (Roche) using LightCycler TaqMan Master mix (Roche).

Table 1. Nucleotide sequences of the primers used for PCR.

Gene		Sequence	Accession number	Probe number
MMP-8	Sence primer	5'-cggggaagacatacttcttcgtaa-3'	NM_022221.1	#67
	Antisence primer	5'-catggatcttctttgattgtcgt-3'		
MMP-9	Sence primer	5'-cctctcgcatgaagacgacataa-3'	NM_031055.1	#42
	Antisence primer	5'-ggtcaggtttagagccacga-3'		
TIMP-1	Sence primer	5'-cagcaaaaggccttcgtaaa-3'	NM_053819.1	#76
	Antisence primer	5'-tggctgaacagggaaacact-3'		

The PCR reaction parameters were as follows: 95℃ for 10 min, 45 cycles of 10 s at 95℃, 30 s at 60℃, and 1 s at 72℃. Fluorescence data were analyzed with LightCycler software (Roche). The mRNA levels were compared to 18 s rRNA as a standard, and relative expression ratios were obtained[24].

CT image acquisition and reconstruction

The computed tomography (CT) studies were performed on 11-month-old rats euthanized by overdose with pentobarbital, using the GE Explore Locus CT system (GE Healthcare UK Ltd., Buckinghamshire, England). X-ray settings were 80 mA and 50 kVp. CT images were visualized using MicroView 2.0 software (GE Healthcare).

Morphological study

The fresh aortic arch was harvested from all rats and stored in saline on ice. It was then dissected from the surrounding tissues and fixed in 10% formalin neutral buffer solution (pH7.4; Wako Pure Chemical Industries, Ltd, Osaka). Sections of the aorta were stained with Oil Red O.

MMP-9 and CD68 immunostaining in the aortic arch

Aortic arch sections (5-μm sections) were dewaxed in xylene and placed on slides in the glass slide chamber, which was filled with processing buffer (citric acid buffer, pH 6.0). Sections were blocked for 1 h in phosphate-buffered saline (PBS) containing 5% normal goat serum (NGS) and then exposed to primary antibody overnight at 4℃, either a rabbit polyclonal antibody (1 : 200) against rat MMP-9 (AB19016, Millipore Co., Billerica, MA) or mouse monoclonal antibody (1 : 200) against rat CD68 (MAB1435, Millipore). The slides were then washed in PBS and incubated with anti-rabbit IgG or anti-mouse IgG secondary antibody. After being rinsed in PBS, tissues were incubated with avidin-biotin complex and visualized with diaminobenzidine (DAB). The sections were finally counterstained with hematoxylin and eosin. Immunostaining of sections was uniformly timed and assessed by microscopy[5].

Table 2. Bw, VisF, EpidF, TC, TG, and HbA1c results for 11-month-old SD-ND, SD-HFDS, SHHR-ND and SHHR-HFDS rats. The results are presented as mean ± S.E.M.

	SD-ND	SD-HFDS	SHHR-ND	SHHR-HFDS
BW (g)	633.4 ± 30.3	699 ± 24.7	600.1 ± 11.5 *	627.8 ± 16.5
VisF (g)	21.0 ± 2.5	31.9 ± 3.1	18.9 ± 0.7 *	53.7 ± 3.4 ##, **, $$
EpidF (g)	14.4 ± 1.6	15.4 ± 1.2	5.6 ± 0.3 ##, $$	8.7 ± 0.4 #, $$
TC (mg / dL)	61.4 ± 9.3	105.0 ± 17.1	211.0 ± 17.4 **, ##	392.6 ± 18.7 ##, **, $$
TG (mg / dL)	89.7 ± 32.4	128.8 ± 17.6	146.8 ± 40.7	300.0 ± 113.4 $$
HbA1c (%)	< 2.5%	< 2.5%	< 2.5%	< 2.5%

*: P < 0.05 vs. SD-HFDS. **: P < 0.001 vs. SD-HFDS. #: P < 0.005 vs. SD-ND.
##: P < 0.001 vs. SD-ND. $$: P < 0.001 vs. SHHR-ND.

Statistical analysis

The statistical significance of the results was determined using one-way analysis of variance (ANOVA) followed by a Bonferroni's test. Correlations were calculated by Pearson's product moment correlation coefficient. All data were expressed as mean ± S.E.M. $P < 0.05$ was considered significant.

Results

Table 2 lists the effects of HFDS on BW, VisF, EpidF, TC, TG and HbA1c in SD-ND, SD-HFDS, SHHR-ND and SHHR-HFDS groups. There was no significant change in BW between the SHHR groups, while BW in the SD groups was increased by HFDS feeding. In both the SD and SHHR groups, VisF weight and EpidF weight were significantly elevated after ingestion of HFDS. Compared to the SD-HFDS, there was a significant elevation in VisF in the SHHR-HFDS, whereas EpidF levels in the SHHR groups were significantly lower than in the SD groups. Plasma HbA1c levels were unchanged among the SD and SHHR groups. CT images of abdominal sections, and VisF photo images, after abdominal dissection in 11-month-old rats, are shown in Fig. 1. VisF in these images is in accordance with the increase in VisF weight. On the other hand, the pannicular on CT images resembles the increase in EpidF weight. SHHRs thus tend to an increased VisF more readily than SD rats.

The levels of oxidative stress in rat plasma are shown in Fig. 2A. The d-ROMs levels in SHHR-HFDS (674.1 ± 22.8 U.CARR) were significantly higher than in SD-ND (297.9 ± 23.1 U.CARR), SD-HFDS (425.1 ± 14.3 U.CARR) and SHHR-ND (322.3 ± 12.8 U.CARR). There was a significantly positive correlation (r = 0.830) between the VisF / BW ratio and d-ROMs in all rats (Fig. 2B). SHHR-HFDS showed a 3.44-fold increase in MMP-9 mRNA expression in WBCs by Q-PCR compared to SD-ND rats (Fig. 3A). Moreover, there was a significantly positive relationship (r = 0.465) between d-ROMs and MMP-9 mRNA in all rats (Fig. 3B). However, the relationship between d-ROMs and MMP-9 mRNA was weak

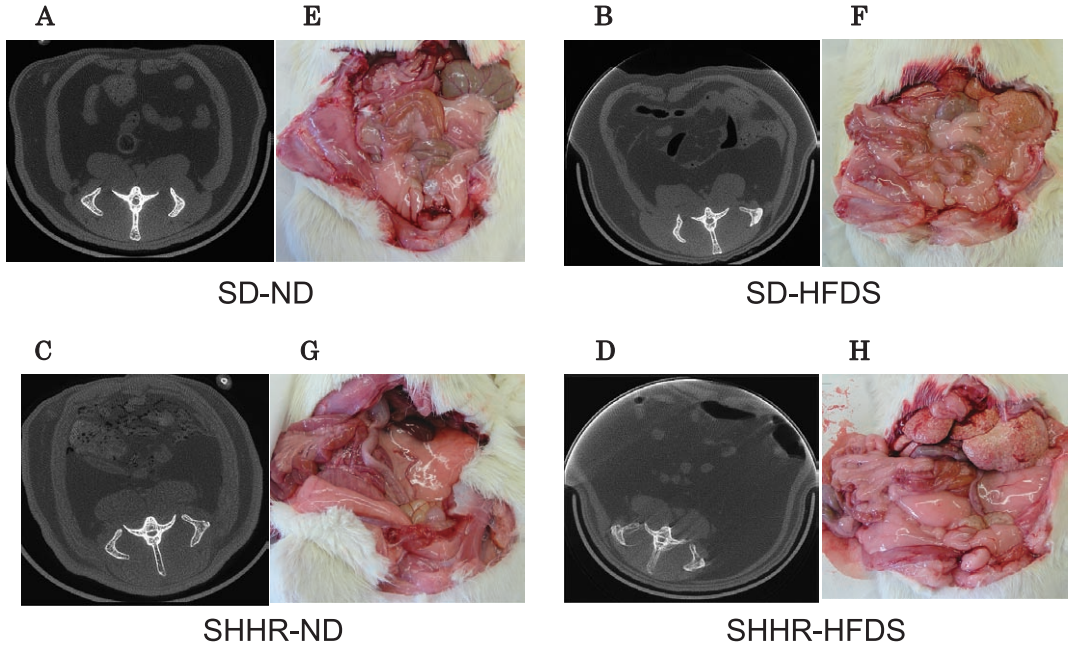

Fig. 1. CT images of abdominal sections and VisF images after abdominal dissection in SD-ND (A, E), SD-HFDS (B, F), SHHR-ND (C, G) and SHHR-HFDS (E, H) at 11 months of age.

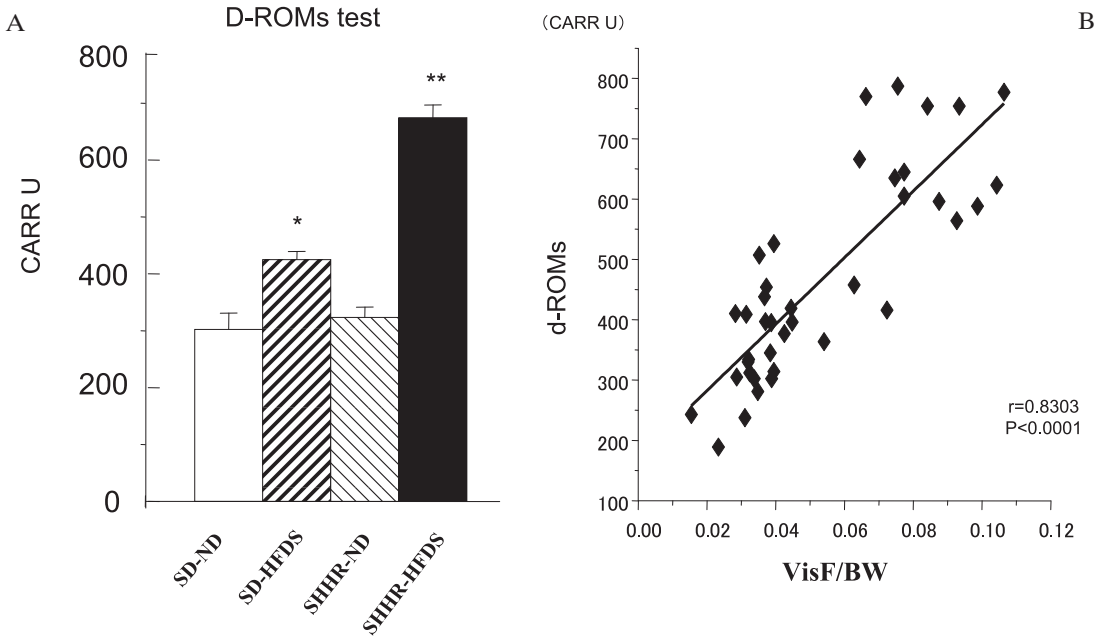

Fig. 2. The graph shows increased d-ROMs test in plasma of SD-ND, SD-HFDS, SHHR-ND and SHHR-HFDS (A). Data are provided as mean ± S.E.M. *: $P < 0.01$ vs. SD-ND and SHHR-ND. **: $P < 0.001$ vs. SD-ND, SD-HFDS and SHHR-ND. A significant positive correlation ($r = 0.83$, $P < 0.001$) between VisF / BW ratio and d-ROMs was observed in all rats (B).

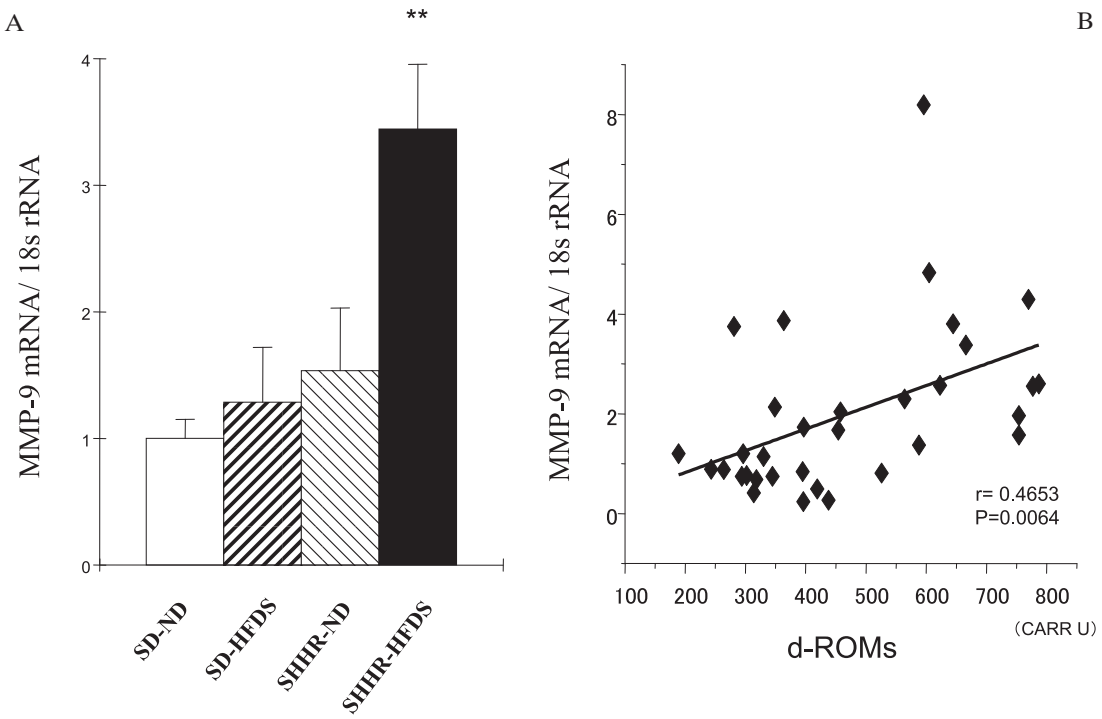

Fig. 3. The graph shows increased MMP-9 mRNA expression in WBCs of SD-ND, SD-HFDS, SHHR-ND and SHHR-HFDS, relative to SD-ND, as determined by Q-PCR (A). Data are provided as mean ±S.E.M. **: $P < 0.001$ vs. SD-ND, SD-HFDS and SHHR-ND. A significant positive correlation ($r = 0.47$, $P < 0.01$) between d-ROMs and MMP-9 mRNA was observed in all rats (B).

compared to the relationship between the VisF / BW ratio and d-ROMs. No significant relationship was observed between the VisF / BW ratio and MMP-9 mRNA (data not shown).

TIMP-1 and MMP-8 mRNA expressions in rat WBCs by Q-PCR are shown in Fig. 4. TIMP-1 in SHHR-ND and SHHR-HFDS decreased to 21% and 46%, respectively, of that in SD-ND. MMP-8 positive WBCs, thought to be polymorphonuclear neutrophil (PMN)-type cells, showed no significant increase in SHHR-HFDS compared to SD-ND.

Morphological analysis of the aorta from SHHRs and SD rats using Oil Red O showed lipid deposition in the subintimal space in both the SHHR-ND and SHHR-HFDS groups (Fig. 5), with the deposition more advanced in the SHHR-HFDS group. Fig. 6 and 7 represent typical images of aorta immunostained for CD68 and MMP-9, respectively. Staining of CD68, a macrophage marker, is clearly apparent at the aortic intima in SHHR-HFDS, while MMP-9 staining was detected mainly at the aortic intima surface. The staining of MMP-9 was almost completely colocalized with CD68 staining and overlapped regions showing lipid deposition.

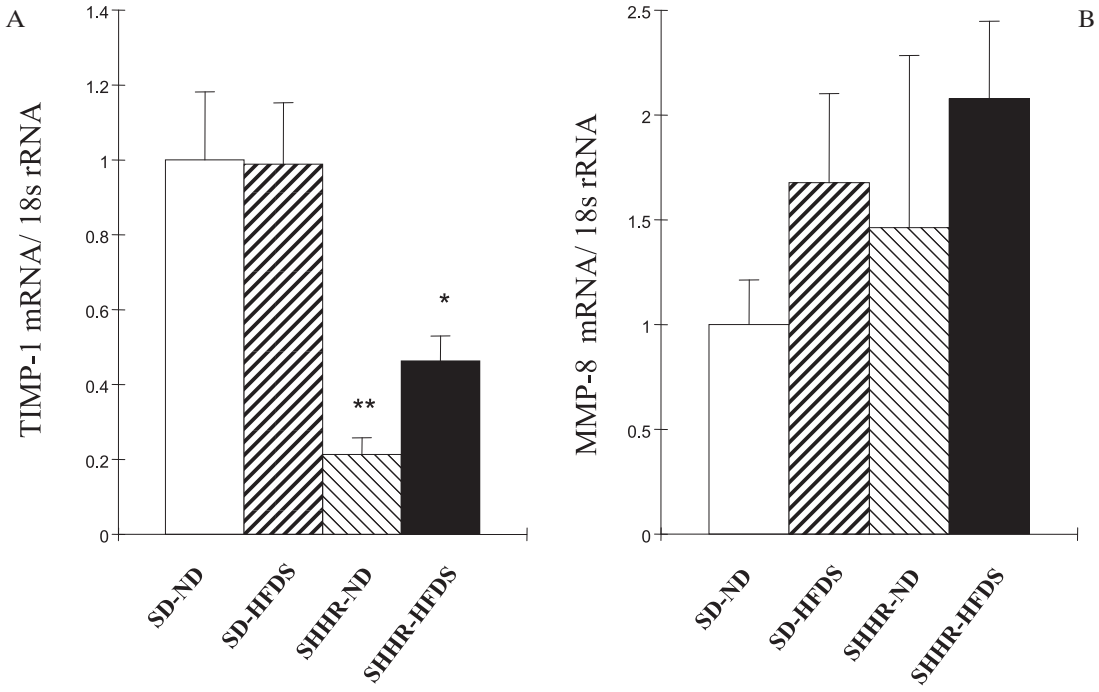

Fig. 4. The graph shows changes in TIMP-1 (A) and MMP-8 (B) mRNA expression in WBCs of SD-ND, SD-HFDS, SHHR-ND and SHHR-HFDS, relative to SD-ND, as determined by Q-PCR. Data are provided as mean±S.E.M. *: $P<0.05$, **: $P<0.01$ vs. SD-ND, SD-HFDS.

Discussion

The present study demonstrated that the increased oxidative stress associated with VisF accumulation increased MMP-9 expression in WBCs of SHHR-HFDS. In addition, the macrophage accumulation and upregulated MMP-9 expression on the aortic intima overlapped the regions of lipid deposition in SHHR-HFDS.

Several previous studies have established a relationship between obesity and lifestyle-related diseases. In particular, VisF obesity is directly correlated with the clustering of lifestyle-related diseases, leading to various vascular diseases[25–27]. Indeed, our atherogenic factor-overlapped animal rats accumulate VisF more easily than normal rats, and in the present study all rat groups showed increased oxidative stress following VisF accumulation. There was also a significantly positive relationship between VisF / BW ratio and oxidative stress in all groups, with the underlying pathological conditions of hypertension and hyperlipidemia having a weak yet direct effect on this relationship. Systemic oxidative stress, measured by urinary 8-epi-PGF2α concentration, was closely associated with visceral fat accumulation in a previous study, and the correlation coefficient for VisF and urinary 8-epi-PGF2α concentration was the highest among all parameters analyzed ($r=0.636$, $p<0.0001$)[28]. Increased oxidative stress is one cause of increased vascular permeability in the visceral fat of obese

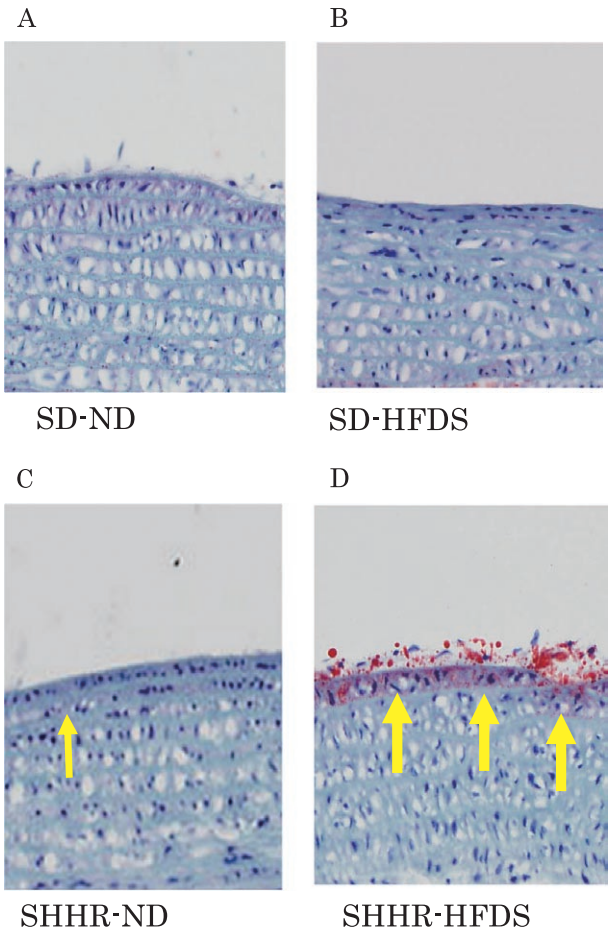

Fig. 5. Histological changes in the aorta of rats at 11 months of age. The aorta tissues (aortic arch) were analyzed by Oil Red O staining in SD-ND (A), SD-HFDS (B), SHHR-ND (C) and SHHR-HFDS (D). Yellow arrows indicate lipid depositions (neutral fat). Original magnification, ×200.

animals[29, 30]. Another proposed cause relates to the abundant synthesis and secretion of angiotensinogen by adipose tissue[31, 32], especially visceral tissue[32, 33]. Angiotensinogen is cleaved by rennin to angiotensin I and then converted to angiotensin II by angiotensin-converting enzyme in adipose tissue. Thus, angiotensin II is also produced locally in adipose tissue[33]. Angiotensin II induces nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression and activation in vascular endothelial cells[34, 35], yielding reactive oxygen species (ROS) as a product[36, 37]. Clearly, visceral fat accumulation is closely associated with increased oxidative stress.

MMP-9 and TIMP-1 play important roles in the progression of atherosclerosis[38, 39] and oxidative stress is associated with MMP-9 expression. Plasma MMP-9 activity is increased in hypertension[40] and in high-fat calorie-induced type 2 diabetes mellitus[41]. In the present study, SHHR-HFDS exhibited a higher MMP-9 expression in WBCs than other rat groups. Furthermore, a positive correlation was found between fractional changes in malondialdehyde, a marker of oxidative stress, and MMP-9 in patients with acute coronary syndrome[12].

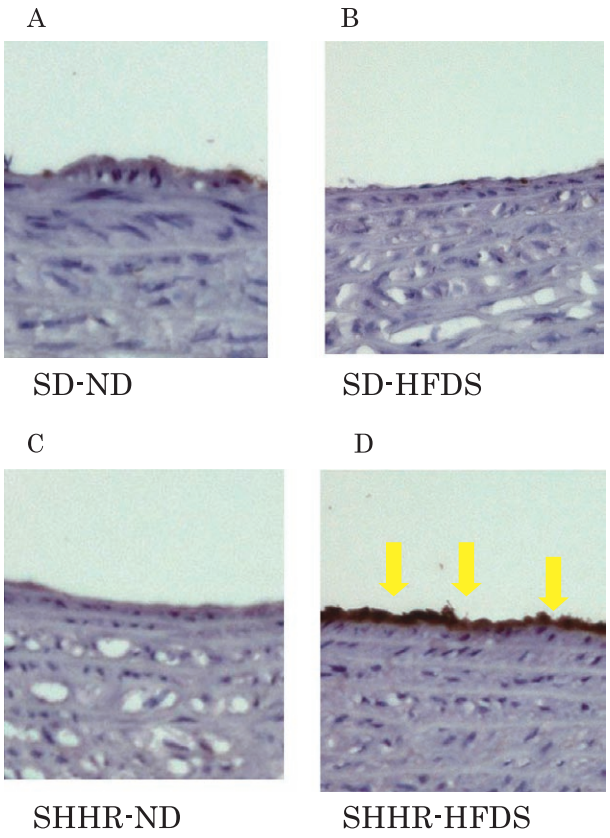

Fig. 6. Immunostaining of CD68 in an aortic cross-section (positive staining is brown). Yellow arrows indicate MMP-9 staining. SD-ND (A), SD-HFDS (B), SHHR-ND (C) and SHHR-HFDS (D). Original magnification, ×200.

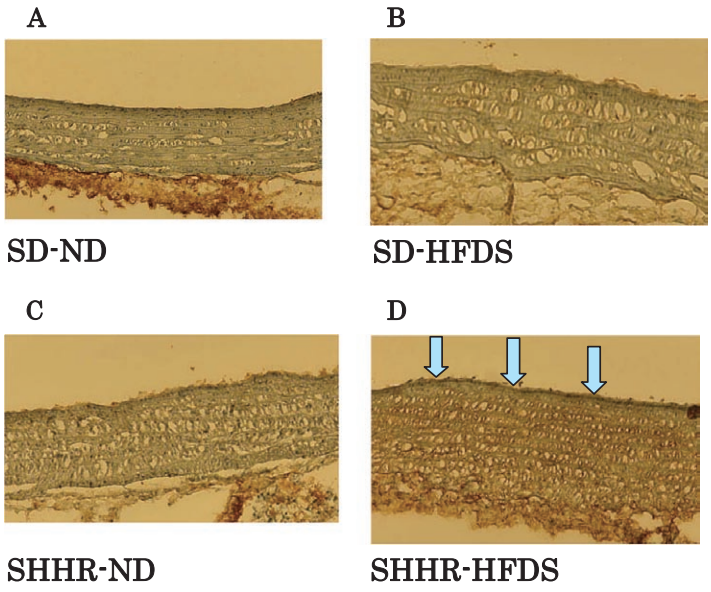

Fig. 7. Immunostaining of MMP-9 in an aortic cross-section (positive staining is brown). White arrows indicate MMP-9 staining. SD-ND (A), SD-HFDS (B), SHHR-ND (C) and SHHR-HFDS (D). Original magnification, ×100.

TIMP-1 is a natural inhibitor of MMP, especially pro-MMP-9, acting to maintain the structural integrity of basement membranes and protects them from degeneration[14]. The relative balance of MMP-9 and TIMP-1 is thus important in approximating the value of absolute MMP-9 activity[15]. TIMP-1 expression is also decreased by hypertension[42], and in the present study, TIMP-1 expression in WBCs was decreased in SHHR groups. Thus, MMP-9 activity would increase in SHHR-HFDS through down-regulated TIMP-1 expression. MMP-8 is released from neutrophils at sites of inflammation and vascular lesions, and is associated with atherosclerosis[43, 44]. In this study, MMP-8 expression in WBCs did not increase significantly ; however, the tendency of MMP-8 expression resembled that of MMP-9 expression. Therefore, MMP-8 activity might also increase significantly with the decrease in TIMP-1 expression[15, 44].

There was substantial lipid deposition in the aorta of SHHR-HFDS, whereas few such areas were observed in other rats. Increased VisF, oxidative stress, and MMP-9 expression correlated with the observed morphological features of the aortic tissue. In addition, the lipid deposition sites overlapped the staining patterns of both MMP-9 and CD68, with the latter marker indicating macrophage accumulation in the aorta[45].

A typical feature of atherosclerotic lesions is the appearance of lipid-laden foam cells. Oxidation of low-density lipoprotein (oxLDL) triggers the generation of a series of oxidation byproducts[46, 47]. The avid uptake of oxLDL by macrophage scavenger receptors leads to the foam cells as well as fatty streak development in the arterial wall, which is one of the earliest stages of atherosclerotic plaque progression[47]. Macrophage MMP's are significant players in the weakening and rupture of these plaques[48], and MMP-9 mRNA expression and activity is high in macrophages[49]. Therefore, MMP-9 is a likely key factor in plaque rapture and the subsequent onset of acute coronary syndrome. Future studies should focus on the association with MMP-9 expression and macrophage accumulation.

In conclusion, the present study suggested that increased oxidative stress generated from visceral fat accumulation induces MMP-9 expression and macrophage accumulation on the aorta intima of atherogenic factor-overlapped animal model rats. The SHHR model may help in the elucidation and treatment of atherosclerosis.

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