suggested that the atherogenic properties of oxLDL affecting endothelial cells are mediated mainly via LOX-1. OxLDL induces endothelial dysfunction/apoptosis, a major change in vascular biology observed at the beginning of atherogenesis through LOX-1-mediated pathway [10]. Furthermore, the binding of ligands to LOX-1 induces the activation of NADPH oxidase [11–13], which leads to oxidative stress in the vessel wall. LOX-1 is dynamically up-regulated by various atherogenic substances such as advanced glycated end products, angiotensin II, and oxLDL itself [14–16]. Furthermore, the expression of LOX-1 is enhanced under various proatherogenic conditions, such as diabetes, hypertension [17], and dyslipidemia [18–20]. Reflecting the proatherogenic properties of LOX-1, disruption of LOX-1 gene in mice actually preserved endothelial function and reduced atherogenesis under hyperlipidemia. Recently, Sankaralingam *et al.* [21] reported the enhanced expression of LOX-1 in association with the condition of preeclampsia.

In the present study, we sought to establish the role of LOX-1 in the arterial wall lipid deposition under hypertension, utilizing SHR-SP. Here, we present the experimental results, which link endothelial dysfunction and vascular lipid retention model via LOX-1.

Materials and methods

Animals

All protocols were approved by the Institutional Animal Care and Use Committee of the National Cardiovascular Center. Eight-week-old male SHR-SP and Wistar–Kyoto rats (WKY) (Japan SLC, Hamamatsu, Japan) were fed with high-fat chow (1.25% cholesterol, 0.5% cholate, 20% milk casein, 15% cocoa butter; α-tocopherol: 2 mg/kg chow) and physiological saline (9 g/l NaCl) instead of drinking water. To evaluate the involvement of LOX-1-mediated pathway, anti-LOX-1 antibody (TS20; 10 mg/kg body weight) or nonimmune mouse IgG (I5381; 10 mg/kg body weight, Sigma-Aldrich, St. Louis, Missouri, USA) was administered via a tail vein two times, just before and on the fourth day of a week's loading. TS20 is the antibody formerly named JTX20 [22]. To some animals indicated in the results, the high-fat diet supplemented with α-tocopherol (50 mg/kg chow) was fed. Body weight and blood pressure were measured before loading and every other day during the examination. Blood pressure was determined by a non-invasive tail cuff and pulse transducer system (BP-98A; Softron, Tokyo, Japan).

After the loading period, the rats were euthanized with inhalation of diethyl ether. The abdominal cavity was immediately opened, and a systemic venous blood was sampled from inferior vena cava. Then, the rat was perfused systemically with physiological saline solution. The mesentery was excised out with intestine. Then, the enteric canal and mesenteric fat and vein were removed to isolate mesenteric artery. Serum levels of total cholesterol (TC), triglyceride, phospholipids, and high-density lipoprotein (HDL) were determined using commercially available reagent kits (Wako, Tokyo, Japan).

Quantitative reverse transcription-polymerase chain reaction

Expression of LOX-1 mRNA in rat mesenteric artery was evaluated by quantitative reverse transcription–polymerase chain reaction (QRT-PCR). Complementary DNA was synthesized with total RNA (1 μg/ml per sample) and random hexamers using SuperScript III RNase H-reverse transcriptase (Invitrogen, Carlsbad, California, USA). Aliquots from each cDNA solution were subjected to QRT-PCR using a primer pair and a quencher specific to rat LOX-1 sequence (Rn00591116) using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, California, USA). A GAPDH fragment was amplified as an internal control. Data are expressed as the ratio of LOX-1 to GAPDH mRNA.

Whole-mount immunohistochemistry

The segments of mesenteric artery were fixed with 2% formalin, and incubated consecutively with 1 mg/ml of dispase/PBS, 2% formalin/0.2% Triton X-100/PBS, methanol at −20°C, and 0.1% Triton X-100 in Tris-buffered saline. After blocking, the tissues were stained with Cy3-labeled antibovine LOX-1 antibody (TS20, 5 μg/ml) or Cy3-labeled mouse IgG as a negative control. The tissues were dehydrated in graded alcohols, and cleared in xylene. A whole-mount immunohistochemical image was obtained by a confocal laser microscope (Fluoview; Olympus, Tokyo Japan).

Oil Red O staining of accumulated lipid in mesenteric arteries

Oil Red O staining was used to detect lipid deposition in mesenteric arteries. The isolated mesenteric artery was fixed with 10% formalin, and washed with PBS. After flushing with 60% isopropanol for 5 min at room temperature, the blood vessel was incubated for 15 min at 55°C with 0.6% Oil Red O (Merck KGaA, Darmstadt, Germany) in 60% isopropanol. Then, the vessel was serially rinsed with 60 and 30% isopropanol, and PBS. The number of lipid deposits was manually counted under the stereomicroscope (Stemi 2000; Carl Zeiss, Göttingen, Germany).

Immunohistochemistry

Freshly frozen cross-sections of mesenteric artery were fixed with 10% formalin. The sections were incubated with 25% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) and 5% donkey serum at 4°C overnight. The sections were incubated with anti-LOX-1 antibody (1 μg/ml, TS20), rabbit anti-oxLDL antiserum (1/100, AB3232; Chemicon, Temecula, California, USA), anti-smooth muscle actin (1 μg/ml, 1A4; DAKO, Carpinteria, California, USA) or anti-rat macrophage (1 μg/ml, RM-4;

TransGenic, Kumamoto, Japan) at 4°C overnight. The sections were then incubated with Biotinylated Link (DAKO), and visualized with Alexa 633 or 546 streptavidin (Invitrogen), or with streptavidin-horseradish peroxidase in combination with diaminobenzidine (DAB).

Measurement of serum LOX-1 ligands

Serum concentration of LOX-1 ligands was determined by sandwich enzyme-linked immunosorbent assay, as described previously [23].

In vivo DiI-oxLDL uptake analysis

Oxidized LDL and DiI-oxLDL were prepared as described previously [9]. One hour after the treatment with anti-LOX-1 antibody (TS20; 10 mg/kg body weight) or mouse IgG (10 mg/kg body weight), SHR-SP were administrated with DiI-oxLDL (10 mg/kg body weight) via tail vein. After 1 h, the whole mesenteric artery was isolated, and fluorescence of the deposited DiI-oxLDL was observed with a macro fluorescence microscope (MVX10; Olympus) equipped with a cooled CCD camera (ORCA-1394-ER; Hamamatsu Photonics, Hamamatsu, Japan). The distribution of DiI-oxLDL was quantitatively estimated by integration of fluorescence of DiI in the vessels. The data are expressed as mean fluorescence intensity (MFI) per unit area of mesenteric artery in the photograph.

Ex-vivo perfusion experiment of isolated mesenteric artery

Segments of mesenteric artery with 100–150 μm internal diameter were isolated in a length of 1–1.5 cm each. The arterial segments were cannulated at both ends and the side branches were ligated. They were then perfused with Krebs-Ringer solution (KRS) (NaCl 155 mmol/l, KCl 3 mmol/l, $CaCl_2$ 2 mmol/l, $MgCl_2$ 1 mmol/l, NaH_2PO_4 3 mmol/l, HEPES 5 mmol/l, glucose 10 mmol/l) containing 10 μg/ml TS20 or control IgG for 30 min, and with the same buffer with or without oxLDL (30 μg/ml) for 30 min. Then, the arterial segments were perfused with 1 μg/ml of DiI-oxLDL/KRS for 15 min, or with 0.1 μg/ml of Evans blue/KRS for 10 min. The vessels were washed with PBS for 5 min and fixed with 4% paraformaldehyde, and subjected to the microscopic analysis. The uptake of DiI-oxLDL or leakage of Evans blue of the arterial segments was semi-quantitatively estimated by integration of fluorescence of DiI or Evans blue in arterial segments. The data are expressed as MFI per unit area of arterial segment in the photograph.

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis between two groups was performed by the Mann–Whitney's *U*-test. Multiple comparisons were done using analysis of variance (ANOVA) with Bonferroni post-hoc analysis. *P* value less than 0.05 was considered as significant.

Results

Enhanced expression of LOX-1 in SHR-SP

QRT-PCR demonstrated that LOX-1 mRNA expression in the mesenteric artery of SHR-SP was 3.5 times higher than that of WKY rats before high-fat diet and saline loading (Fig. 1a). Whole-mount immunostaining using Cy3-labeled anti-LOX-1 antibody further showed abundant LOX-1 expression in SHR-SP, whereas only negligible expression was observed in WKY rats (Fig. 1b).

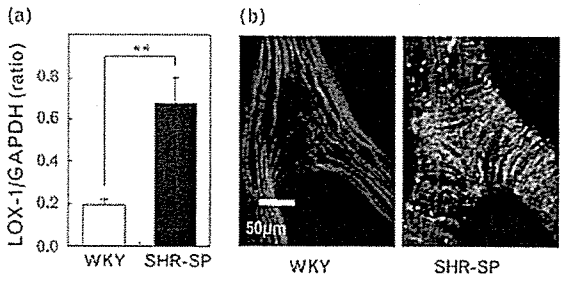
High-fat and salt diet-induced lipid deposition in SHR-SP

Loading with high-fat diet and saline dramatically induced lipid deposition in the mesenteric arteries of SHR-SP, whereas no deposition was observed in control WKY rats. Interestingly, the lipid deposition observed in mesenteric artery was regionally localized and dotted throughout the branches (Fig. 2a). High-fat diet and saline loading resulted in elevation of total serum cholesterol in both rat strains, whereas arterial blood pressure was increased in SHR-SP, but not in WKY. Lipid deposition was observed in SHR-SP in as early as 1 week of high-fat diet and saline loading, whereas WKY showed negligible lipid deposition (Fig. 2b). Thus, the mesenteric arteries of SHR-SP were highly susceptible to lipid deposition.

Relationship of LOX-1 expression with lipid accumulation

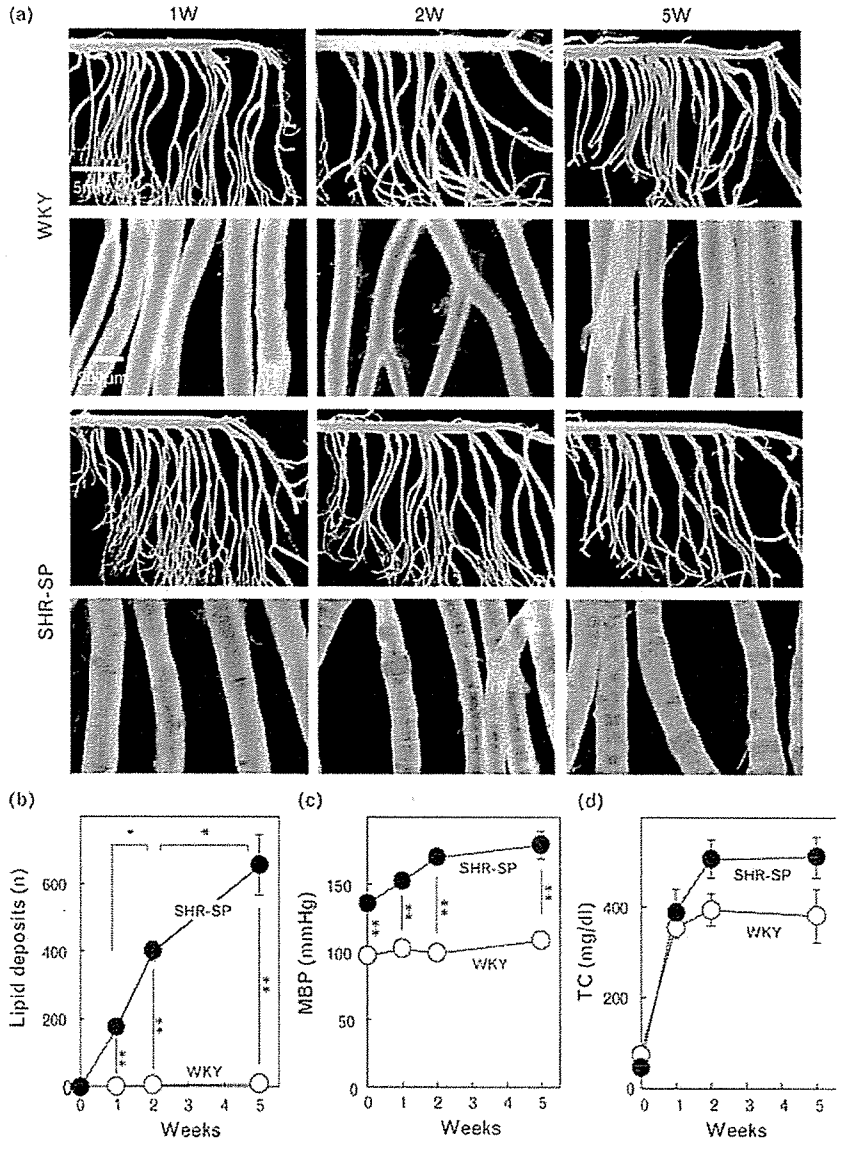
Next, we investigated spatial relationship between LOX-1 and oxLDL in the mesenteric arteries. OxLDL was accumulated in medial smooth muscle layer (Fig. 3a), and associated with LOX-1 expression (Fig. 3b). The expression of LOX-1 was observed in endothelium as well as smooth muscle cells (Fig. 3b-d). Immunoreactivity of macrophages was not observed in these lesions (Fig. 3e).

Fig. 1



Enhanced expression of LOX-1 in mesenteric arteries of SHR-SP. (a) Comparison of LOX-1 mRNA expression in mesenteric arteries between SHR-SP and WKY prior to high-fat diet and saline loading ($n = 11$, $^{**}P < 0.005$). (b) LOX-1 protein expression in mesenteric artery of SHR-SP and WKY rats detected by anti-LOX-1 antibody. Orange and green fluorescence represent the expression of LOX-1 and auto-fluorescence of internal elastic layer, respectively. LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; SHR-SP, stroke-prone spontaneously hypertensive rats; WKY, Wistar–Kyoto rats.

Fig. 2



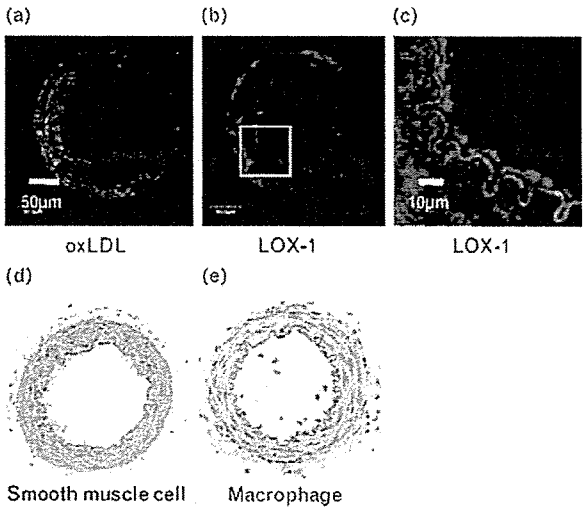
Lipid deposition in mesenteric artery of SHR-SP and WKY after high-fat (HF) diet and saline loading. (a) Oil Red O staining of lipid deposition in mesenteric artery of SHR-SP and WKY after 1, 2 and 5 weeks of HF diet and saline loading. Each upper panel shows the whole branches of mesenteric artery at low magnification and each lower panel shows arteries at high magnification. (b) Time-dependent increase in the number of vascular lipid deposits during HF diet and saline loading. (c) Comparison of mean blood pressure (MBP) between SHR-SP and WKY after HF diet and saline loading. Mean blood pressure of SHR-SP was significantly higher compared with WKY. (d) Comparison of serum cholesterol between SHR-SP and WKY after HF diet and saline loading. The levels of total serum cholesterol in both groups were increased by HF diet and saline loading ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.005$). SHR-SP, stroke-prone spontaneously hypertensive rats; TC, total cholesterol; WKY, Wistar–Kyoto rats.

Suppression of lipid accumulation by the inhibition of LOX-1

The aforementioned histological findings prompted us to investigate whether LOX-1 mediates the vascular lipid accumulation in SHR-SP. Lipid deposition in SHR-SP was significantly reduced by treatment with anti-LOX-1 antibody (Fig. 4a, b). Blood pressure was not significantly different between the two groups (Fig. 4c), although TC concentration notably increased in the group of anti-LOX-1 antibody treatment (Fig. 4d).

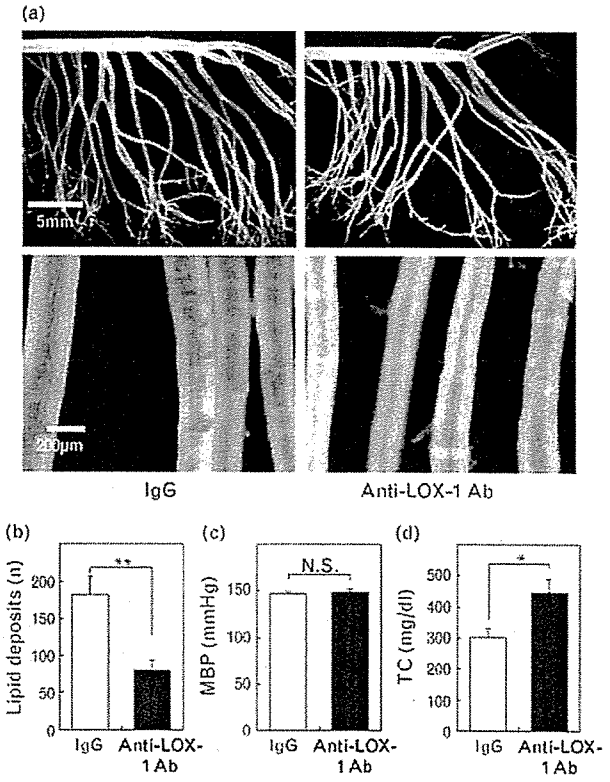
To clarify the significance of oxidative stress, the effects of vitamin E, a lipophilic antioxidant, were examined. As shown in Fig. 5a and b, vitamin E potently suppressed vascular lipid deposition. Concomitantly, the increase in serum levels of LOX-1 ligands induced by a high-fat diet

Fig. 3



Immunostaining analysis of the frozen section at a fat deposit in mesenteric artery of SHR-SP after one week of HF diet and saline loading. (a) Accumulation of oxLDL detected by anti-oxLDL antiserum. OxLDL accumulation was visualized with Alexa546-streptavidin (orange). (b) Expression of LOX-1 detected by anti-LOX-1 antibody. LOX-1, visualized with Alexa633-streptavidin (blue) was expressed in medial smooth muscle layer and intima. Green fluorescence indicates auto fluorescence of the internal elastic layer. (c) Image in high magnification of the yellow square area of (b). The expression of LOX-1 was observed in endothelial cells as well as smooth muscle layer. (d, e) Immunochemical staining of smooth muscle (d) and macrophages (e) of serial cryosection visualized with DAB (brown). Nuclei were counter-stained with Mayers hematoxyline. DAB, diaminobenzidine; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; oxLDL, oxidized low-density lipoprotein; SHR-SP, stroke-prone spontaneously hypertensive rats.

Fig. 4



Effects of anti-LOX-1 antibody on vascular lipid deposition induced by high-fat diet and saline loading. (a) Suppression of vascular lipid deposition by anti-LOX-1 antibody. (b) Effects of anti-LOX-1 antibody on the number of lipid deposits. (c) Effects of anti-LOX-1 antibody on arterial blood pressure. (d) Effects of anti-LOX-1 antibody on serum cholesterol concentration ($n = 11$, $^{*}P < 0.05$, $^{**}P < 0.005$). LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; MBP, mean blood pressure; TC, total cholesterol.

was also significantly reduced by vitamin E (Fig. 5e). Taken together, functional blocking of LOX-1 or reduction in oxidative stress efficiently suppressed the arterial lipid deposition. It is noteworthy that the levels of arterial blood pressure were not significantly reduced by the treatment of anti-LOX-1 antibody, or vitamin E.

Enhanced uptake of oxLDL in mesenteric artery of SHR-SP

The involvement of LOX-1 in lipid accumulation was further examined by analyzing the distribution of DiI-oxLDL. DiI-oxLDL administered intravenously to SHR-SP was regionally taken up in mesenteric artery (Fig. 6a, b). This acute distribution of DiI-oxLDL was suppressed by pretreatment with anti-LOX-1 antibody (Fig. 6a, b). These findings indicate that the oxLDL distributed via LOX-1-mediated pathway possibly contributes to localized lipid deposition in the vessel wall.

To further examine the accumulation of oxLDL via LOX-1-mediated pathway, ex-vivo perfusion experiment was also performed. Perfusion of isolated SHR-SP mesenteric artery with DiI-oxLDL induced accumulation of DiI-oxLDL in the vessel wall (Fig. 6c, d, control). Pretreatment with oxLDL increased the accumulation of DiI-oxLDL, which was suppressed with anti-LOX-1 antibody (Fig. 6c, d, oxLDL).

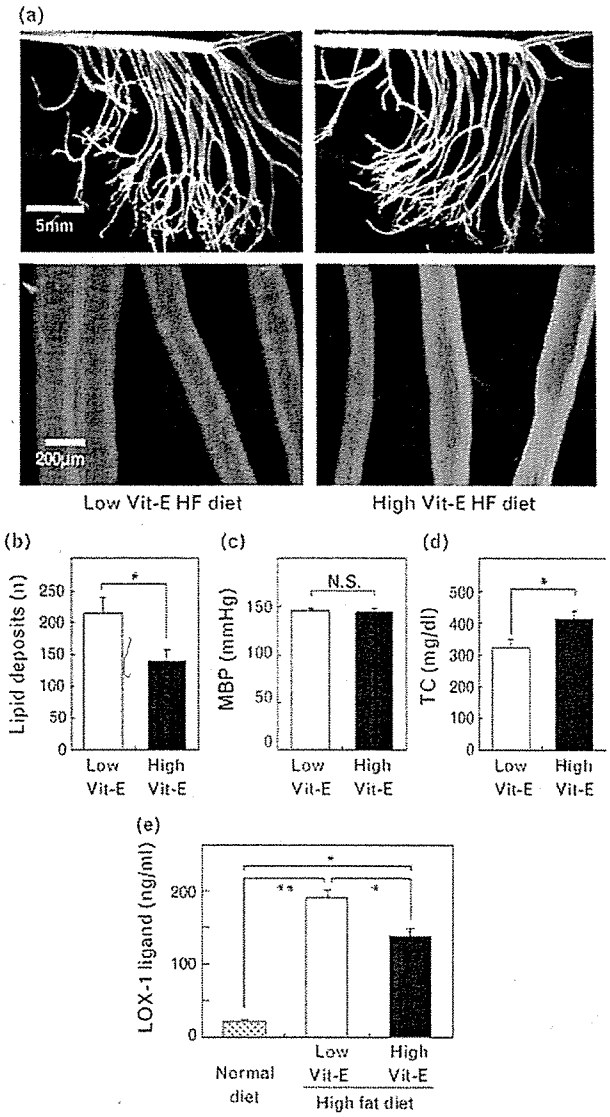
Enhancement of vascular permeability via LOX-1-mediated pathway

Next, the regulation of vascular permeability via LOX-1 pathway was evaluated by exudation of Evans blue in mesenteric artery. *Ex vivo* perfusion experiment demonstrated that the pretreatment with oxLDL increased exudation of Evans blue from luminal surface of the isolated mesenteric arteries of SHR-SP, which was suppressed by anti-LOX-1 antibody (Fig. 7a, b).

Discussion

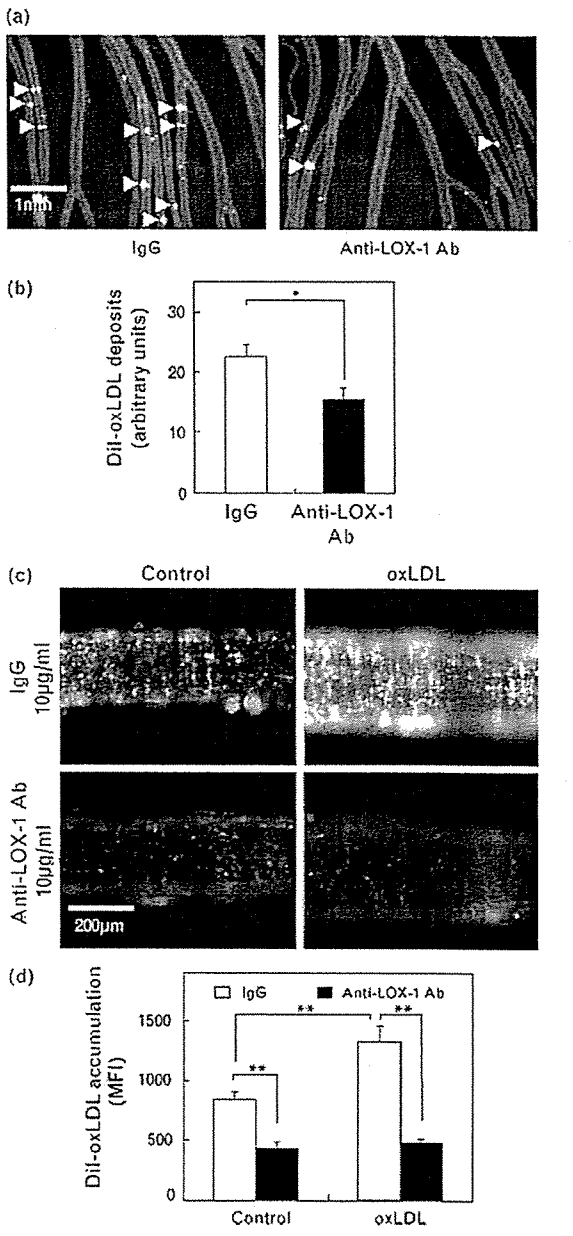
In the hypertensive state, mechanical stress induced by hemodynamic forces such as shear stress and stretch force is one of the most important factors contributing to

Fig. 5



Effects of vitamin E on vascular lipid accumulation. (a) Suppressive effects of high dose of vitamin E on lipid deposition induced by high-fat diet and salt loading. (b) Quantitative analysis of vitamin E effects on vascular lipid deposition induced by high-fat diet and salt loading. (c) Effects of vitamin E on arterial blood pressure. Vitamin E exerted no influence on mean blood pressure. (d) Effects of vitamin E on serum cholesterol levels. Administration of high dose of vitamin E increased total serum cholesterol levels. (e) Changes in LOX-1 ligand by high-fat diet containing low or high dose of vitamin E ($n=6$, $^{*}P<0.05$, $^{**}P<0.005$). LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; MBP, mean blood pressure; TC, total cholesterol.

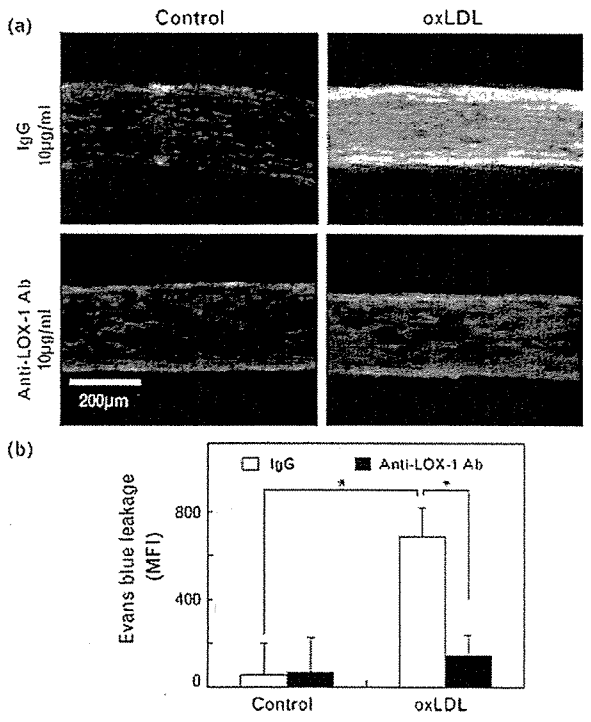
Fig. 6



Distribution of Dil-oxLDL to mesenteric arteries *in vivo*. (a) Suppressive effects of anti-LOX-1 antibody on Dil-oxLDL accumulation (arrowhead) in mesenteric artery *in vivo*. (b) Quantitative analysis of anti-LOX-1 antibody effect on the number of Dil-oxLDL accumulation foci *in vivo* ($n=6$, $^{*}P<0.05$). (c) Accumulation of Dil-oxLDL in isolated mesenteric artery from SHR-SP. The vessels were pretreated with (right) or without oxLDL (left) in the presence of anti-LOX-1 antibody (lower) or IgG (upper). (d) Quantitative analysis of Dil-oxLDL accumulation in mesenteric artery in (c) ($n=8$, $^{**}P<0.005$). LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; oxLDL, oxidized low-density lipoprotein; SHR-SP, stroke-prone spontaneously hypertensive rats.

endothelial dysfunction/injury. Derangement of humoral factors, caused by enhanced activity of renin-angiotensin and sympathetic nervous systems, and increase in oxidative stress further make the pathophysiology of hypertension more complicated. Combination of high-fat diet

Fig. 7



Enhancement of permeability in mesenteric artery via oxLDL-LOX-1 pathway. (a) Effects of oxLDL on permeability of isolated mesenteric artery observed by Evans blue leakage. The vessels were pretreated with anti-LOX-1 antibody (lower) or IgG (upper). (b) Quantitative analysis of the vascular permeability of mesenteric artery in (a) ($n = 6$, $^*P < 0.05$). LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; oxLDL, oxidized low-density lipoprotein.

with salt loading to SHR-SP is a suitable and appropriate experimental model for the investigation of cellular and molecular mechanisms of the earliest process of atherosclerosis under hypertension and hyperlipidemia. Employing this model here, we demonstrated that LOX-1 mediates vascular lipid retention and augmentation of vascular permeability. high-fat diet induced characteristic localized lipid accumulation in mesenteric arteries of SHR-SP. This vascular lipid accumulation was associated with the augmented expression of LOX-1 and suppressed by the treatment of anti-LOX-1 antibody. These findings indicate that LOX-1 enhances oxLDL retention to the vessel wall. Conversely, oxLDL enhanced vascular permeability via LOX-1, leading to further lipid retention. Thus, LOX-1 might play a critical role in acute atherosis under hypertension. This vicious cycle under enhanced expression of LOX-1 may explain the molecular mechanisms of the response-to-retention model of atherosclerosis.

Anti-LOX-1 antibody, when administered *in vivo*, neither reduced blood pressure nor serum cholesterol concentration. LOX-1 expression is enhanced in SHR-SP and salt-loaded Dahl salt-sensitive hypertension rats [17], and reduction of blood pressure by either a calcium channel blocker or hydralazine effectively suppresses the expression of LOX-1 [24]. These findings suggest that LOX-1 system might be in the downstream cascade of hypertension. Enhanced expression of LOX-1 under hypertensive state is further likely to promote lipid deposition if hyperlipidemia coexists with hypertension. Indeed, hypertension and hypercholesterolemia have synergistic deleterious effects on coronary endothelial function in association with augmented expression of LOX-1 [25].

In hyperlipidemic rabbits, ApoB-containing LOX-1 ligands, presumably regarded as oxLDL, are accumulated in the plasma and atherosclerotic lesions [26]. Reflecting the elevated plasma levels of LOX-1 ligands, the expression of LOX-1 in endothelium of hyperlipidemic rabbits is augmented. This observation is explained by the experimental results that oxLDL itself induces the expression of LOX-1 in endothelial cells [19]. In the present investigation, the levels of LOX-1 ligands including oxLDL were increased by high-fat diet and reduced by vitamin E. Vascular lipid accumulation was also suppressed by vitamin E. From these findings, we speculate that enhanced expression of LOX-1 in the vasculature associated with elevated levels of LOX-1 ligands in the blood stream might be an important driving mechanism for atherogenesis. Indeed, direct blockade against LOX-1 significantly prevented lipid accumulation in arterial wall, demonstrating that the above hypothesis actually worked at least in the present hypertension model.

It is of note that anti-LOX-1 antibody reduced the enhanced vascular permeability in SHR-SP. Ex-vivo permeability analysis using Evans blue demonstrated that oxLDL augments vascular permeability via LOX-1 mediated pathway. Yamori *et al.* have suggested that vascular permeability might be an instigating mechanism preceding lipid deposition in vascular smooth muscle cells of SHR-SP. Thus, anti-LOX-1 antibody exerts its effects on lipid deposition both by suppression of vascular permeability in endothelium and by blocking of the uptake of lipids in smooth muscle cells.

Limitation of the study

Acute atherosis observed in SHR-SP is not precisely equal to atherosclerosis; rather, it is similar to vascular changes in human preeclampsia. The accumulation of macrophages was not observed in the present model even in the later stage, but smooth muscle cells incorporate lipids instead to transform into smooth muscle-derived foam cells. The present study focuses on elucidation of the role of LOX-1 in the process of lipid retention, and the issue of macrophage accumulation needs to wait for further study.

Cellular lipid uptake and foam cell formation are mediated by various pathway other than LOX-1, such as CD-36 and scavenger receptor-A. Previous investigations reported that compared with Apo E knockout mice those lacking SR-A or CD36 showed marked reduction in atherosclerotic lesion area [27,28]. In the present study, we focused on the role of LOX-1, and did not evaluate the involvement of these other pathways.

In summary, we have clarified that LOX-1 enhances vascular permeability and retention of lipids including oxLDL under the hypertensive state. Since oxLDL up-regulates expression of LOX-1, the vicious cycle composed of LOX-1, vascular permeability, and lipid retention might occur in the initial step of atherogenesis. Hence, the oxLDL-LOX-1 pathway may work in both endothelial dysfunction and the responses-to-lipid retention models.

Acknowledgements

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There are no conflicts of interest.

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Original article

Allogeneic administration of fetal membrane-derived mesenchymal stem cells attenuates acute myocarditis in rats

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ABSTRACT

We reported previously that the autologous administration of bone marrow-derived mesenchymal stem cells (BM-MSC) significantly attenuated myocardial dysfunction and injury in a rat model of acute myocarditis by stimulating angiogenesis and reducing inflammation. Because BM aspiration procedures are invasive and can yield low numbers of MSC after processing, we focused on fetal membranes (FMs) as an alternative source of MSC to provide a large number of cells. We investigated whether the allogeneic administration of FM-derived MSC (FM-MSC) attenuates myocardial injury and dysfunction in a rat myocarditis model. Experimental autoimmune myocarditis (EAM) was induced in male Lewis rats by injecting porcine cardiac myosin. Allogeneic FM-MSC obtained from major histocompatibility complex-mismatched ACI rats (5×10^5 cells/animal) were injected intravenously into Lewis rats one week after myosin administration. At day 21, severe cardiac inflammation and deterioration of cardiac function were observed. The allogeneic administration of FM-MSC significantly attenuated inflammatory cell infiltration and monocyte chemoattractant protein 1 expression in the myocardium and improved cardiac function. In a T-lymphocyte proliferation assay, the proliferative response of splenic T lymphocytes was significantly lower in cells obtained from FM-MSC-treated EAM rats that reacted to myosin than in cells obtained from vehicle-treated rats with EAM. T-lymphocyte activation was significantly reduced by coculture with FM-MSC. The allogeneic administration of FM-MSC attenuated myocardial dysfunction and inflammation, and the host cell-mediated immune response was attenuated in a rat model of acute myocarditis. These results suggest that allogeneic administration of FM-MSC might provide a new therapeutic strategy for the treatment of acute myocarditis.

1. Introduction

Acute myocarditis is a nonischemic heart disease characterized by myocardial inflammation and edema. This disease is associated with rapidly progressing heart failure, arrhythmia, and sudden death [1,2]. Although the early evidence showing the efficacy of immunoglobulin and interferon therapies appears promising, these results have yet to be demonstrated in randomized controlled clinical trials. The current options are restricted to supportive care for patients with heart failure and arrhythmia. The lack of a specific treatment and the potential severity of the illness emphasize the importance of new effective therapeutic strategies for myocarditis.

Mesenchymal stem cells (MSC) are multipotent stem cells present in the bone marrow (BM), adipose tissue, and many other tissues, and these cells can differentiate into a variety of cells, including

Abbreviations: MSC, mesenchymal stem cells; BM, bone marrow; BM-MSC, bone marrow-derived mesenchymal stem cells; FMs, fetal membranes; FM-MSC, fetal membrane-derived mesenchymal stem cells; MHC, major histocompatibility complex; ACI, August–Copenhagen–Irish; GFP, green fluorescent protein; EAM, experimental autoimmune myocarditis; PBS, phosphate-buffered saline; α-MEM, α-minimal essential medium; FBS, fetal bovine serum; TGF-β3, transforming growth factor-β3; FITC, fluorescein isothiocyanate; LVSP, left ventricular systolic pressure; LVDs, left ventricular systolic dimension; LVDd, left ventricular diastolic dimension; H&E, hematoxylin and eosin; MCP1, monocyte chemoattractant protein 1; HRP, horseradish peroxidase.

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adipocytes, osteocytes, chondrocytes, endothelial cells, and myocytes [3–5]. MSC are a promising cell source for regenerative therapies. We have reported that the autologous administration of BM- or adipose tissue-derived MSC improves cardiac function in rat models of dilated cardiomyopathy and myocardial infarction [6–8]. We also recently demonstrated that the administration of autologous BM-derived MSC (BM-MSC) attenuates myocardial injury and dysfunction in rats with acute myocarditis [9].

However, there are limitations to the application of autologous BM in clinical situations. BM procurement procedures in humans may be painful and may yield low numbers of MSC after processing. An alternative source of MSC that could provide large quantities of cells would be advantageous. To address this issue, we focused on fetal membranes (FMs), which are generally discarded as medical waste after delivery, as an alternative source of autologous MSC. Several studies have reported that human FMs contain multipotent cells similar to BM-MSC, which are easy to expand [10,11]. We demonstrated recently that the allogeneic transplantation of FM-derived MSC (FM-MSC) and BM-MSC induces therapeutic angiogenesis in a rat hind-limb ischemia model [12]. MSC have been reported to induce immune tolerance [13,14], and we confirmed that the transplantation of FM-MSC did not elicit any lymphocyte proliferative response despite their allogeneic origin.

In this study, we investigated whether the intravenous allogeneic administration of FM-MSC improves cardiac function and decreases myocardial inflammation in rats with myosin-induced myocarditis, and the mechanisms underlying the changes induced by allogeneic FM-MSC administration.

2. Materials and methods

2.1. Animals

Different strains of rats were used, based on their major histocompatibility complex (MHC) antigen disparities: Lewis rats (MHC haplotype: RT-$1A^l$; Japan SLC, Hamamatsu, Japan), and August–Copenhagen–Irish (ACI) rats (MHC haplotype: RT-$1A^a$; Japan SLC). Green fluorescent protein (GFP)-transgenic Lewis rats (Institute of Laboratory Animals, Kyoto University, Japan) were also used to investigate the distribution of the transplanted FM-MSC. Adult rats, aged 8–12 weeks, were used for the induction of experimental autoimmune myocarditis (EAM) and were maintained in our animal facility. The experimental protocols were approved by the Animal Care Committee of the National Cardiovascular Center Research Institute.

2.2. Preparation of FM-MSC

The isolation and expansion of FM-MSC were performed as described previously [12]. In brief, pregnant ACI rats (15 days postconception) were sacrificed, and their uteri were harvested and placed in phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA, USA). We chose 15 days postconception as the day of FM retrieval because that point was the best in terms of cell isolation and reproducibility in rats. After separation from the placenta, the FMs were minced with scissors and digested with type II collagenase solution (300 U/mL; Worthington Biochemicals, Lakewood, NJ, USA) for 1 h at 37 °C in a shaking water bath. Enzyme activity was neutralized with α-minimal essential medium (α-MEM; Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen). After filtration through a mesh filter (100 μm; BD Biosciences, Bedford, MA, USA) and centrifugation at 300×g for 5 min, the dissociated FM cells were suspended in α-MEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen), plated onto 100-mm uncoated culture dishes, and incubated at 37 °C in 5% CO_2. The population of adherent, spindle-shaped MSC was expanded. Almost all of non-adherent cells were hematocytes in the morphological observation. The isolation of FM-MSC was repeated three times to evaluate its reproducibility. In all experiments, the FM-MSC were used at passages 5–7.

2.3. Differentiation of FM- and BM-MSC into adipocytes, osteocytes, and chondrocytes

The multipotency of FM-MSC was assessed as described previously [12]. FM-MSC were seeded into six-well plates, and the differentiation into adipocytes and osteocytes was induced at 40–50% confluence. To induce differentiation into adipocytes, MSC were cultured with adipocyte differentiation medium: 0.5 mM 3-isobutyl-1-methylxanthine (Wako Pure Chemical Industries, Osaka, Japan), 1 μM dexamethasone (Wako Pure Chemical Industries), 50 μM indomethacin (Wako Pure Chemical Industries), and 10 μg/mL insulin (Sigma-Aldrich, St. Louis, MO) in α-MEM. After two weeks of differentiation, adipocytes were identified by the existence of lipid vesicles stained with oil red O (Sigma-Aldrich).

To induce differentiation into osteocytes, MSC were cultured in α-MEM with MSC osteogenesis supplements (Dainippon Sumitomo Pharma, Osaka, Japan), according to the manufacturer's instructions. After two weeks of differentiation, osteocytes were identified by the existence of mineral nodule deposition stained with alizarin red S (Sigma-Aldrich).

To induce differentiation into chondrocytes in three-dimensional culture, the pellet culture method was used. MSC were centrifuged at 150×g for 5 min and resuspended at a density of 1×10^6 cells/mL in an hMSC Differentiation BulletKit-Chondrogenic (Cambrex Bio Science, Walkersville, MD) supplemented with transforming growth factor-β3 (TGF-β3; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, 5×10^5 cells were placed in a 15-mL polypropylene tube and centrifuged at 150×g for 5 min. Fresh medium was added every third day. After three weeks of differentiation, cell pellets were fixed with 4% paraformaldehyde and embedded in paraffin. Differentiation into chondrocytes was identified by the existence of proteoglycan deposition stained with Safranin O (Sigma-Aldrich). Differentiation of FM-MSC was repeated three times to evaluate reproducibility.

2.4. Flow cytometry

Cultured FM-MSC were analyzed by flow cytometry (FACSCalibur, BD Biosciences) as described previously [12]. Fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies against rat CD34 (clone ICO-115, Santa Cruz Biotechnology, Santa Cruz, CA), CD45 (clone OX-1, BD Biosciences), CD73 (clone 5F/B9, BD Biosciences), CD90 (clone OX-7, BD Biosciences), RT$1A^{a,b,l}$ (clone B5, BD Biosciences), and RT1B (clone OX-6, BD Biosciences) were used. Isotype-identical antibodies served as controls. Flow cytometric analysis of FM-MSC was repeated three times to evaluate reproducibility.

2.5. Acute myocarditis model

Purified cardiac myosin was prepared from the ventricular muscles of pig hearts according to a previously described procedure [15]. The antigen was dissolved at a concentration of 20 mg/mL in PBS containing 0.3 M KCl and mixed with an equal volume of complete Freund's adjuvant containing 11 mg/mL *Mycobacterium tuberculosis* (Difco Laboratories, Sparks, MD, USA). The rats were anesthetized with an intraperitoneal injection of 20 mg/kg sodium pentobarbital, and 0.1 mL of the antigen–adjuvant emulsion was injected into each footpad.

Forty-five Lewis rats were assigned randomly into the following three groups and treated with: 1) 0.2 mL of PBS only (Sham group,

$n=15$); 2) 0.2 mL of cardiac myosin only (MyoC group, $n=15$); and 3) 0.2 mL of cardiac myosin and FM-MSC (MyoC + FM-MSC group, $n=15$). One week after the myosin injection, allogeneic FM-MSC (5×10^5 cells/animal) or vehicle (PBS) was administered intravenously via the tail vein. We chose an intravenous route for the administration of FM-MSC because of its clinical applicability. We chose the time for the cell injection of one week after myosin injection on the basis of our previous report showing that this time was the most effective compared with other schedules [9]. In this study, we used the 5×10^5 cell administration because this cell number did not elicit the significant and persistent rise in pulmonary arterial pressure.

To assess the distribution of the injected cells, FM-MSC (5×10^5 cells/animal) derived from GFP-transgenic Lewis rats were administered intravenously via the tail vein seven days after the myosin injection. One day, one week or four weeks after the injection of the cells, the rats were sacrificed, and sections of tissues were obtained from the heart, lung, spleen, and liver, and embedded in paraffin ($n=4$ for each tissue).

2.6. Hemodynamic studies

Hemodynamic studies were performed on day 21 after the myosin injection. Anesthesia was maintained with isoflurane (1.5–2.0 vol.% in air), and a polyethylene catheter (model PE-50, BD Biosciences) was placed into the left ventricle through the right carotid artery. The heart rate was monitored by electrocardiography. Heart rate, mean arterial pressure, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure, maximum dP/dt, and minimum dP/dt were used as the hemodynamic indices and were recorded simultaneously during ventilation after a minimum equilibration period of 20 min. The hemodynamic studies were performed with the investigators blinded to the treatment group and the analyses were performed offline.

2.7. Echocardiographic studies

Echocardiography was performed on day 21 after the myosin injection. The rats were anesthetized with isoflurane (1.5–2.0 vol.% in air). A 12-MHz probe was placed at the left fourth intercostal space for M-mode imaging using two-dimensional echocardiography (Sonos 5500, Philips, Bothell, WA, USA). The left ventricular systolic dimension (LVDs), left ventricular diastolic dimension (LVDd), anterior wall thickness, posterior wall thickness, and ejection fraction were measured and recorded as the average for three beats. Fractional shortening (%) was calculated as $([LVDd-LVDs]/LVDd)\times100$. The echocardiography studies were performed with the investigators blinded to the treatment group and the analyses were performed offline.

2.8. Histopathological studies

The hearts were excised above the origin of the great vessels on day 21 after the myosin injection, and the heart and body weight were recorded. The heart, spleen, pancreas, kidney, and liver were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned to 4-μm thickness, and stained with hematoxylin and eosin (H&E) or Masson's trichrome. H&E-stained sections were evaluated by a cardiovascular pathologist (H.I.-U.) with no knowledge of the experimental groups, who characterized the myocardial injury and inflammation using the following scale: 0, absent or questionable presence; 1, limited focal distribution; 2–3, intermediate severity; and 4, coalescent and extensive foci throughout the entire transversely sectioned ventricular tissue. To evaluate fibrosis, the collagen volume fraction was analyzed with image-processing software (Win ROOF, Mitani Co. Ltd., Tokyo, Japan).

2.9. Immunohistochemical studies

Deparaffinized sections were incubated with Protein Block (DakoCytomation, Glostrup, Denmark) and then with mouse anti-rat CD68 (clone ED-1; Millipore, Bedford, MA, USA), CD3 (BD Biosciences), monocyte chemoattractant protein 1 (MCP1; BD Biosciences), or rabbit anti-GFP antibody (Invitrogen) in diluent for 40 min, followed by incubation with horseradish peroxidase (HRP)-linked rabbit anti-mouse IgG or DakoCytomation Envision+ System-HRP Labeled Polymer (DakoCytomation) for 30 min. The sections were visualized with 0.5% diaminobenzidine (DakoCytomation) and 0.03% hydrogen peroxide, and counterstained with hematoxylin. Five random fields from each rat were photographed (Biorevo BZ-9000; Keyence, Osaka, Japan). The numbers of CD68- and CD3-positive cells and the MCP1-positive areas were analyzed with the image-processing software. The number of GFP-positive cells was counted in 20 randomly selected fields per section.

2.10. T-lymphocyte proliferation assay

T lymphocytes were isolated from the spleens of rats with myocarditis on day 21 using a previously described procedure [12]. The responder T lymphocytes were isolated from untreated control Lewis rats (sham-TL), myosin-treated Lewis rats with myocarditis (MyoC-TL), or allogeneic FM-MSC-administered myosin-treated Lewis rats with myocarditis (MyoC + FM-MSC-TL). The responder T lymphocytes (1×10^5 cells/well) were cultured in a 96-well culture plate with 50 μg/mL of purified porcine heart myosin (Sigma-Aldrich) as the stimulator, with or without the modulator cells. The modulator cells were allogeneic FM-MSC obtained from MHC-mismatched ACI rats (1×10^5 cells/well). When no allogeneic FM-MSC were added, T lymphocytes isolated from normal Lewis rats were added to adjust the cell number (1×10^5 cells/well). The modulator cells were irradiated at 30 Gy before they were cultured. A total of 2×10^5 cells were cocultured in 0.2 mL of tissue culture medium (RPMI 1640; Invitrogen) supplemented with 20% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in 96-well flat-bottomed plates for three days. The proliferation of the responding cells was assessed using the Cell Proliferation Biotrak ELISA System (GE Healthcare, Piscataway, NJ, USA), according to the manufacturer's instructions. T-lymphocyte proliferation is presented as the percentage of the relative proliferation response, as follows: % change in proliferative response = $(\text{absorbance}_{\text{each group}}/\text{absorbance}_{\text{sham-TL without modulator}})\times100$.

2.11. Statistical analysis

Data are expressed as mean ± standard error of the mean. Analysis of variance was used to compare each variable between groups, and the post hoc Tukey test was used to locate the significant differences. Differences were considered significant at $P<0.05$.

3. Results

3.1. Distribution of intravenously administered FM-MSC in rats with myocarditis

We obtained FM-MSC from ACI rats (MHC haplotype: RT-1A^a) to examine whether the allogeneic administration of FM-MSC could attenuate myocarditis in Lewis rats (MHC haplotype: RT-1A^l). FM-MSC differentiated into adipocytes, osteocytes, and chondrocytes ($n=3$, each) (Figs. 1(B)–(D)). Flow cytometric analysis of cultured FM-MSC at passage 5 ($n=3$) demonstrated that FM-MSC were positive for CD73, CD90, and MHC class I (i.e., RT1A) but were negative for CD34, CD45, and MHC class II (i.e., RT1B) (Fig. 1(E)).

To investigate the distribution of the intravenously injected FM-MSC in the rats with myocarditis, we intravenously administered

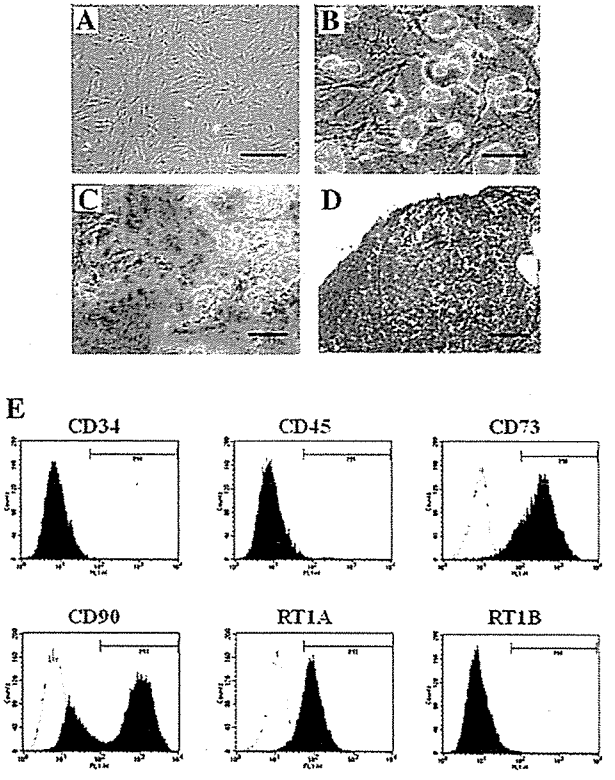

Fig. 1. Characterization of FM-MSC. (A) Morphology of FM-MSC. Scale bar = 100 μm. (B–D) Multipotency of FM-MSC. FM-MSC differentiated into adipocytes (B), osteocytes (C), and chondrocytes (D) ($n=3$ each). Scale bars = 50 μm. (E) Flow cytometric analysis of FM-MSC ($n=3$ each).

GFP-expressing FM-MSC obtained from GFP-transgenic Lewis rats one week after the myosin injection. GFP immunostaining demonstrated that GFP-positive transplanted FM-MSC were present in the heart, lungs, spleen, and liver at both one day and one week after FM-MSC injection (Fig. 2(A)). Semiquantitative analysis demonstrated that a significant number of GFP-positive cells were observed in the lung (day 1, 4.0 ± 0.4 cells/mm^2; and day 7, 2.4 ± 0.4 cells/mm^2), whereas only a few GFP-expressing engrafted cells were observed in the other organs at day 1 (heart, 1.1 ± 0.5 cells/mm^2; spleen, 0.3 ± 0.2 cells/mm^2; and liver, 0.2 ± 0.1 cells/mm^2) and one week (heart, 0.7 ± 0.3 cells/mm^2; spleen, 0.8 ± 0.1 cells/mm^2; and liver, 1.0 ± 0.5 cells/mm^2) after the FM-MSC injection ($n=4$ for each) (Fig. 2(B)). We found no GFP-positive cells four weeks after the FM-MSC injection.

3.2. Improvement in cardiac function by allogeneic administration of FM-MSC

All rats with myocarditis survived the 21-day observation period. On day 21, the heart weight/body weight ratio was significantly lower in the MyoC + FM-MSC group than in the MyoC group (3.7 ± 0.1 vs 4.3 ± 0.1, $P<0.05$). Hemodynamic analysis revealed significant improvements in the MyoC + FM-MSC group compared with the MyoC group in LVSP (119.1 ± 3.4 vs 102.0 ± 4.3 mmHg), mean arterial pressure (84.3 ± 3.2 vs 69.0 ± 3.1 mmHg), and maximum dP/dt (8202 ± 516 vs 6445 ± 373 mmHg/s) ($P<0.05$ for all; $n=15$ in each group) (Figs. 3(A) and (B), and Table 1).

Echocardiographic analysis revealed significant improvements in the MyoC + FM-MSC group compared with the MyoC group in LVDs (3.8 ± 0.2 vs 4.7 ± 0.1 mm), fractional shortening (45.4 ± 1.8 vs $36.8\pm1.2\%$), and ejection fraction (83.2 ± 1.6 vs $74.2\pm1.4\%$) ($P<0.05$ for all). Wall thickness was also significantly thinner in the MyoC + FM-MSC group than in the MyoC group (anterior wall thickness diastole, 2.1 ± 0.2 vs 2.5 ± 0.2 mm; and posterior wall thickness diastole, 2.2 ± 0.1 vs 2.5 ± 0.1 mm) ($P<0.05$ for all; $n=15$ in each group) (Figs. 3(C)–(E) and Table 2).

3.3. Attenuation of myocardial inflammation after allogeneic administration of FM-MSC

Histological analysis on day 21 after the induction of experimental myocarditis showed severe myocardial inflammatory changes. The semiquantitative grading of H&E-stained heart sections by a pathologist (H.I.-U.) using a blinded method showed significantly lower in the MyoC + FM-MSC group than in the MyoC group for tissue granulation (2.7 ± 0.2 vs 3.1 ± 0.1), eosinophil infiltration (1.8 ± 0.1

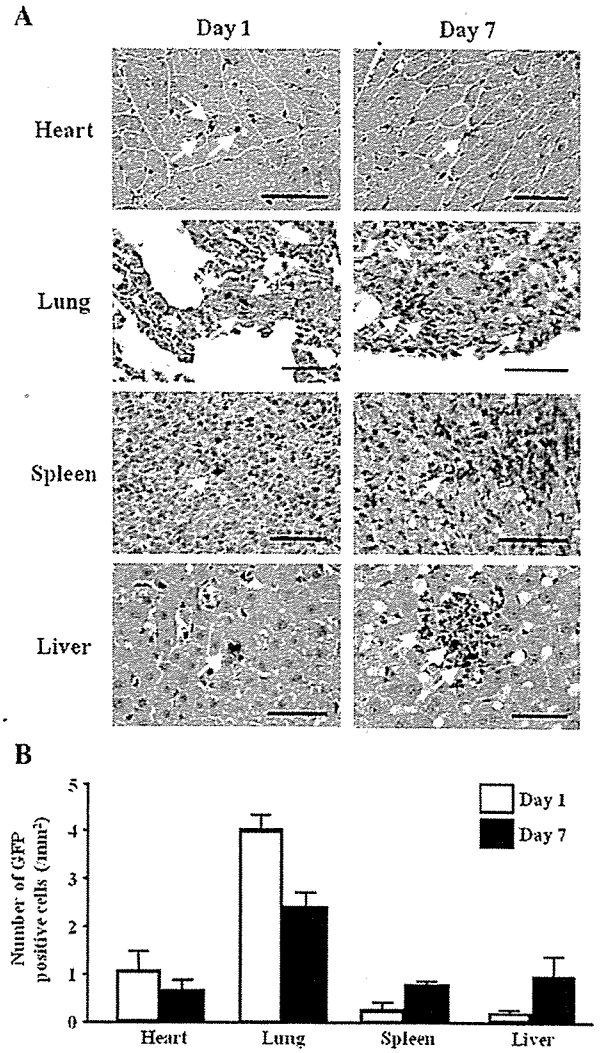

Fig. 2. Distribution of intravenous-administered FM-MSC in acute myocarditis. (A) GFP-positive-administered FM-MSC were present in the heart, lung, spleen, and liver one day and one week after cell administration (brown stain; yellow arrows). Scale bars = 50 μm. (B) Semiquantitative analysis demonstrated that a significant number of GFP-positive cells were observed in the lung, whereas only a few GFP-expressing engrafted cells were observed in the other organs at one day and one week after the FM-MSC injection ($n=4$ for each organ).

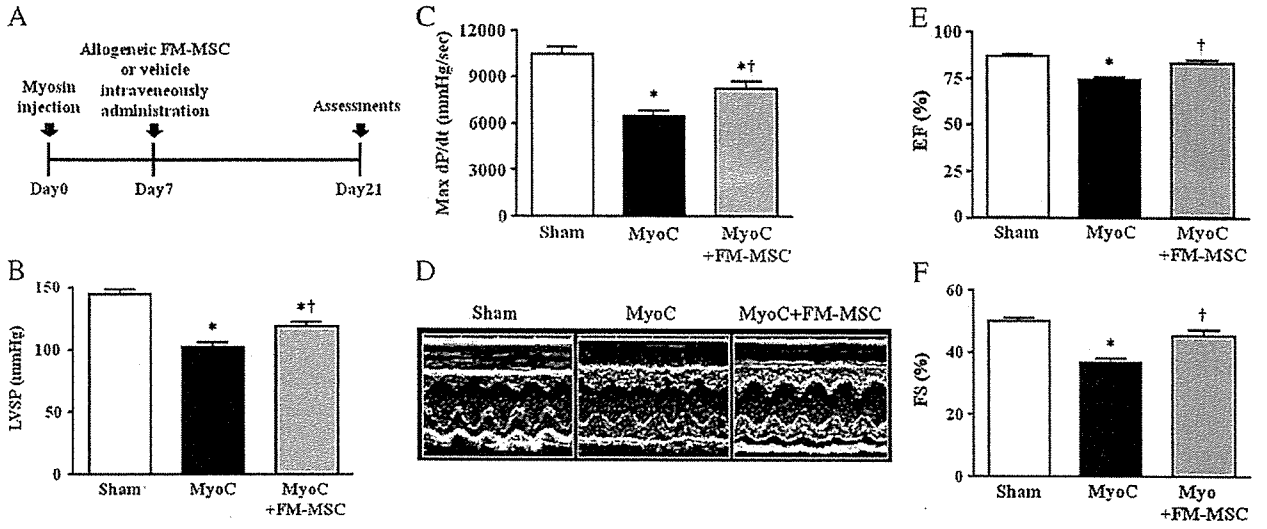

Fig. 3. Effects of administration of allogeneic FM-MSC on hemodynamic and echocardiographic parameters in acute myocarditis. (A) The study flowchart. (B) Left ventricular systolic pressure (LVSP) and (C) maximum dP/dt (max dP/dt) were measured in sham-treated rats given vehicle (Sham group), myosin-treated rats given vehicle (MyoC group), and myosin-treated rats given FM-SC (MyoC + FM-MSC group; $n = 15$ in each group). (D) Representative echocardiographic images showing wall thickening and poor movement in the MyoC group, and improved cardiac contractility in the MyoC + FM-MSC group. (E, F) Allogeneic FM-MSC administration significantly improved the ejection fraction (EF) and fractional shortening (FS; $n = 15$ for each group). Data are expressed as mean ± SEM. *$P<0.05$ vs the Sham group; †$P<0.05$ vs the MyoC group.

vs 2.7 ± 0.1), and edema (2.4 ± 0.1 vs 3.1 ± 0.1) ($P<0.05$ for all; $n = 15$ in each group) (Figs. 4(A) and (B)).

Masson's trichrome staining of the myocardium derived from the MyoC group on day 21 demonstrated prominent and diffuse interstitial fibrosis, which was attenuated dramatically in the MyoC + FM-MSC group (Fig. 4(C)). Quantitative assessment of myocardial fibrosis showed that the Masson's trichrome-stained collagen volume fraction was significantly smaller in the MyoC + FM-MSC group than in the MyoC group (3.1 ± 1.4 vs 7.7 ± 1.9%) ($P<0.05$; $n = 15$ in each group) (Fig. 4(D)).

Immunohistochemical analysis of the myocardial tissue on day 21 showed significantly attenuated infiltration of CD68-positive monocytes/macrophages in the MyoC + FM-MSC group compared with the MyoC group (3713 ± 426 vs 6528 ± 590 cells/mm^2) ($P<0.05$; $n = 15$ in each group) (Figs. 5(A) and (B)).

Immunohistochemical staining of the myocardial tissue for MCP1 on day 21 showed a few positive cells in the myocardial interstitium of the normal heart (Fig. 5(C)). In the rats with myocarditis, increased MCP1 expression was observed in the myocardial interstitium and in the vascular wall of the heart tissue. The hearts of the MyoC + FM-MSC group showed a partial reduction in MCP1 expression. Quantitative analysis demonstrated less MCP1 expression in the MyoC + FM-MSC group than in the MyoC group (0.85 ± 0.1 vs 1.46 ± 0.2%) ($P<0.05$; $n = 15$ in each group) (Fig. 5(D)).

Table 1
Physiological parameters in the three experimental groups.

	Sham	MyoC	MyoC + FM-MSC
HW/BW (g/kg)	2.5 ± 0	4.3 ± 0.1*	3.7 ± 0.1*,**
HR (bpm)	371.9 ± 7.0	337.9 ± 7.6*	364.3 ± 8.5**
MAP (mmHg)	100.1 ± 2.7	69.0 ± 3.1*	84.3 ± 3.2*,**
LVEDP (mmHg)	4.5 ± 1.0	5.7 ± 0.8	6.5 ± 1.0
Min dP/dt (mmHg/s)	−8258.9 ± 422.2	−4980.5 ± 278.6*	−6135.6 ± 375.9*

Sham, sham-operated rats given vehicle; MyoC, myosin-treated rats given vehicle; MyoC + FM-MSC, myosin-treated rats given FM-MSC (5×10^5 cells/animal); FM-MSC, fetal membrane-derived mesenchymal stem cells; HW/BW, heart weight-to-body weight ratio; HR, heart rate; MAP, mean arterial pressure; LVEDP, left ventricular end-diastolic pressure; Min dP/dt, minimum dP/dt.
* $P<0.05$ vs the Sham group.
** $P<0.05$ vs the MyoC group.

3.4. Attenuation of T-cell infiltration by allogeneic administration of FM-MSC

Marked T-cell infiltration was demonstrated by CD3 immunostaining of the cardiac tissues of the MyoC group on day 21. By contrast, T-cell infiltration was attenuated significantly in the MyoC + MSC group (2014 ± 196 vs 3068 ± 455 cells/mm^2) ($P<0.05$ vs the MyoC group; $n = 15$ in each group) (Figs. 5(E) and (F)).

3.5. Suppression of T-lymphocyte activation by allogeneic treatment with FM-MSC demonstrated in a T-lymphocyte proliferation assay

To examine whether allogeneic FM-MSC suppress T-lymphocyte activation, we performed a T-lymphocyte proliferation assay [12]. T lymphocytes collected on day 21 from the MyoC group with myocarditis were cocultured with porcine heart myosin and with irradiated T lymphocytes derived from normal Lewis rats. The proliferative response was significantly higher in the MyoC group with myocarditis than in the sham group T lymphocytes (183.2 ± 2.5 vs 100.0 ± 2.6%, $P<0.05$; $n = 8$ in each group). However, on day 21, the proliferative response was significantly lower in the T lymphocytes derived from the MyoC + FM-MSC group with myocarditis (164.2 ± 0%) than in the MyoC group ($P<0.05$, $n = 8$). T-cell activation was significantly reduced when T lymphocytes from the MyoC group or MyoC + FM-MSC group were cocultured with allogeneic ACI-derived

Table 2
Echocardiographic findings in the three experimental groups.

	Sham	MyoC	MyoC + FM-MSC
LVDs (mm)	3.6 ± 0.1	4.5 ± 0.1*	3.8 ± 0.2**
LVDd (mm)	7.1 ± 0.1	7.2 ± 0.1	7.0 ± 0.2
AWT diastole (mm)	1.4 ± 0	2.5 ± 0.2*	2.1 ± 0.2*,**
PWT diastole (mm)	1.7 ± 0	2.5 ± 0.1*	2.2 ± 0.1*,**

Sham, sham-operated rats given vehicle; MyoC, myosin-treated rats given vehicle; MyoC + FM-MSC, myosin-treated rats given FM-MSC (5×10^5 cells/animal); FM-MSC, fetal membrane-derived mesenchymal stem cells; LVDs, left ventricular systolic dimension; LVDd, left ventricular diastolic dimension; AWT, anterior wall thickness; PWT, posterior wall thickness.
* $P<0.05$ vs the Sham group.
** $P<0.05$ vs the MyoC group.

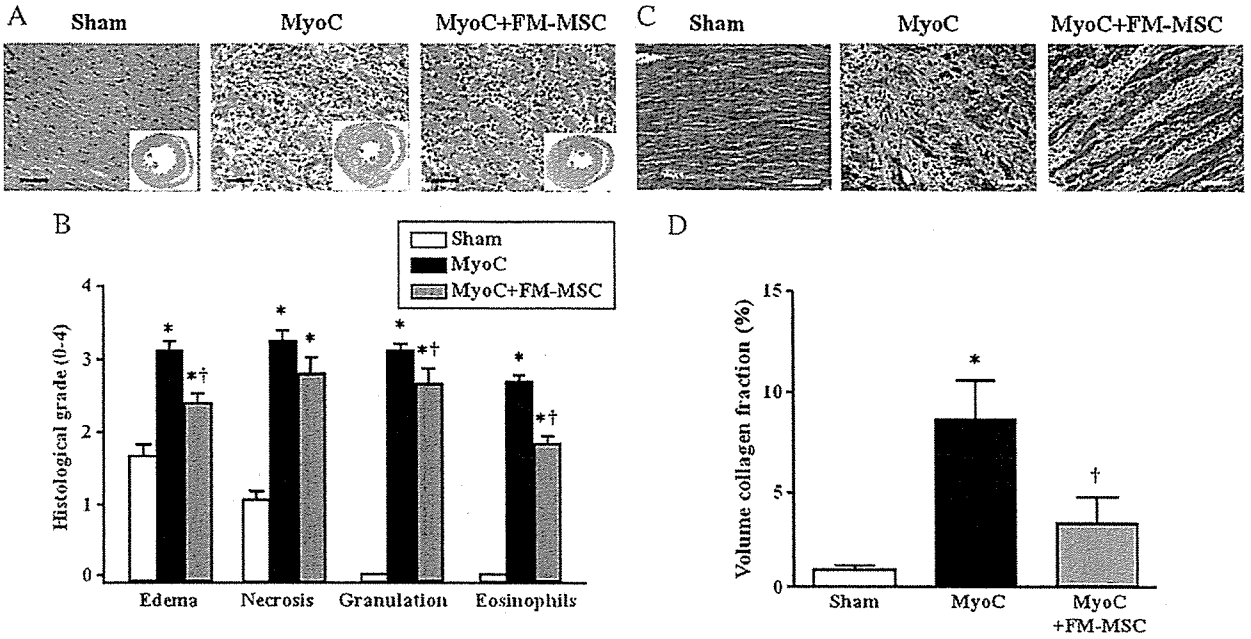

Fig. 4. Histopathological changes of acute myocarditis induced by administration of allogeneic FM-MSC. (A) Myocardial sections show markedly less inflammation in the rats given allogeneic FM-MSC than in the MyoC group. Insets are transverse sections of the myocardium. (B) The semiquantitative histological grading of edema and eosinophil infiltration were markedly lower in the MyoC + FM-MSC group than in the MyoC group ($n = 15$ in each group). (C) Myocardial fibrosis was markedly lower in the allogeneic FM-MSC group than in the MyoC group. (D) The semiquantitative area of fibrosis was smaller in the MyoC + FM-MSC group than in the MyoC group ($n =$ for 15 each group). Scale bars = 50 μm. Data are expressed as mean ± SEM. *$P<0.05$ vs the Sham group; †$P<0.05$ vs the MyoC group.

FM-MSC instead of autologous normal Lewis T cells; the values were 145.1 ± 4.6% for MyoC-TL with allogeneic FM-MSC vs 183.2 ± 2.5% for MyoC-TL without allogeneic FM-MSC and 136.2 ± 3.6% for MyoC + MSC-TL with allogeneic FM-MSC vs 164.2 ± 3.7% for MyoC + MSC-TL without allogeneic FM-MSC ($P<0.05$, $n = 8$ in each group) (Fig. 6). However, in the sham-TL group, proliferation did not differ in the presence or absence of allogeneic FM-MSC. These results show that allogeneic FM-MSC had both an acute suppressive effect on T-lymphocyte proliferation in vitro and a chronic suppressive effect on T-lymphocyte proliferation in the pathological studies.

4. Discussion

In this study, we investigated the therapeutic potential of allogeneic FM-MSC in the acute phase of myocarditis. In our rat model of acute myocarditis, intravenous allogeneic administration of FM-MSC one week after the myosin injection significantly improved cardiac function and the pathological findings in the heart three weeks after the myosin injection. The pathological findings included the attenuated expression of the proinflammatory factor MCP1 and a reduction in the infiltration of T cells and macrophages into the hearts treated with FM-MSC. The T-lymphocyte proliferation assay demonstrated that splenic lymphocytes from rats with myocarditis treated with FM-MSC reacted to myosin less strongly than did the splenic lymphocytes from untreated rats with myocarditis.

Experimental autoimmune myocarditis (EAM) is induced by immunization with cardiac myosin in Lewis rats and is characterized by severe myocardial dysfunction and the appearance of multinucleated giant cells. EAM has been used as an animal model of human giant cell myocarditis [15,16]. This myocarditis model is triphasic and comprises an antigen-priming phase on days 0–13, an autoimmune response phase on days 14–21, and a reparative phase thereafter, which is associated with a chronically dilated cardiomyopathy phenotype. Although the pathogenesis of EAM has not been clarified fully, severe inflammatory cell infiltration is a characteristic of the disease [15,16].

In a recent study, we tried to determine whether MSC improve the function and pathological findings in the affected heart in EAM. The intravenous administration of autologous BM-MSC one week after myosin injection significantly attenuated the myocardial dysfunction in rats with acute myocarditis [9]. Moreover, the intramyocardial transplantation of autologous BM-MSC five weeks after the myosin injection, corresponding to the reparative phase, improved cardiac function in a model of chronic dilated cardiomyopathy [7]. These findings suggest that autologous BM-MSC are an attractive source of cells for transplantation. However, there are several limitations in using an autologous cell source, including their invasiveness, inadequate cell numbers, and donor-site morbidity [17]. In addition, a waiting period is required for transplantation of autologous BM-MSC to prepare an adequate number of cells. Because acute myocarditis is often associated with rapidly progressive heart failure, time-consuming transplantation of autologous BM-MSC is not suitable. Human FMs, which are generally discarded as medical waste after delivery, have been shown recently to be rich sources of MSC [18,19]. Previous reports have suggested that FM-MSC might have regenerative potential for cell therapy. Using a rat hind-limb ischemia model, we demonstrated that allogeneic FM-MSC and autologous BM-MSC are suitable cell sources for tissue regeneration [12]. We infer that, as an alternative source to autologous BM-MSC, allogeneic FM-MSC might be a promising candidate for the cell therapy-based treatment of acute myocarditis.

In this study, intravenous allogeneic administration of FM-MSC significantly improved cardiac function and the pathological findings in the hearts exhibiting EAM. The extent of the improvement was in the range of 30–60% in the indices of the dysfunction level, which are equivalent to those observed in our previous study of administration of autologous BM-MSC [9]. Our previous report focused on the angiogenesis effects and the paracrine action of MSC in EAM, and we focused on the immunomodulatory properties of MSC in this study.

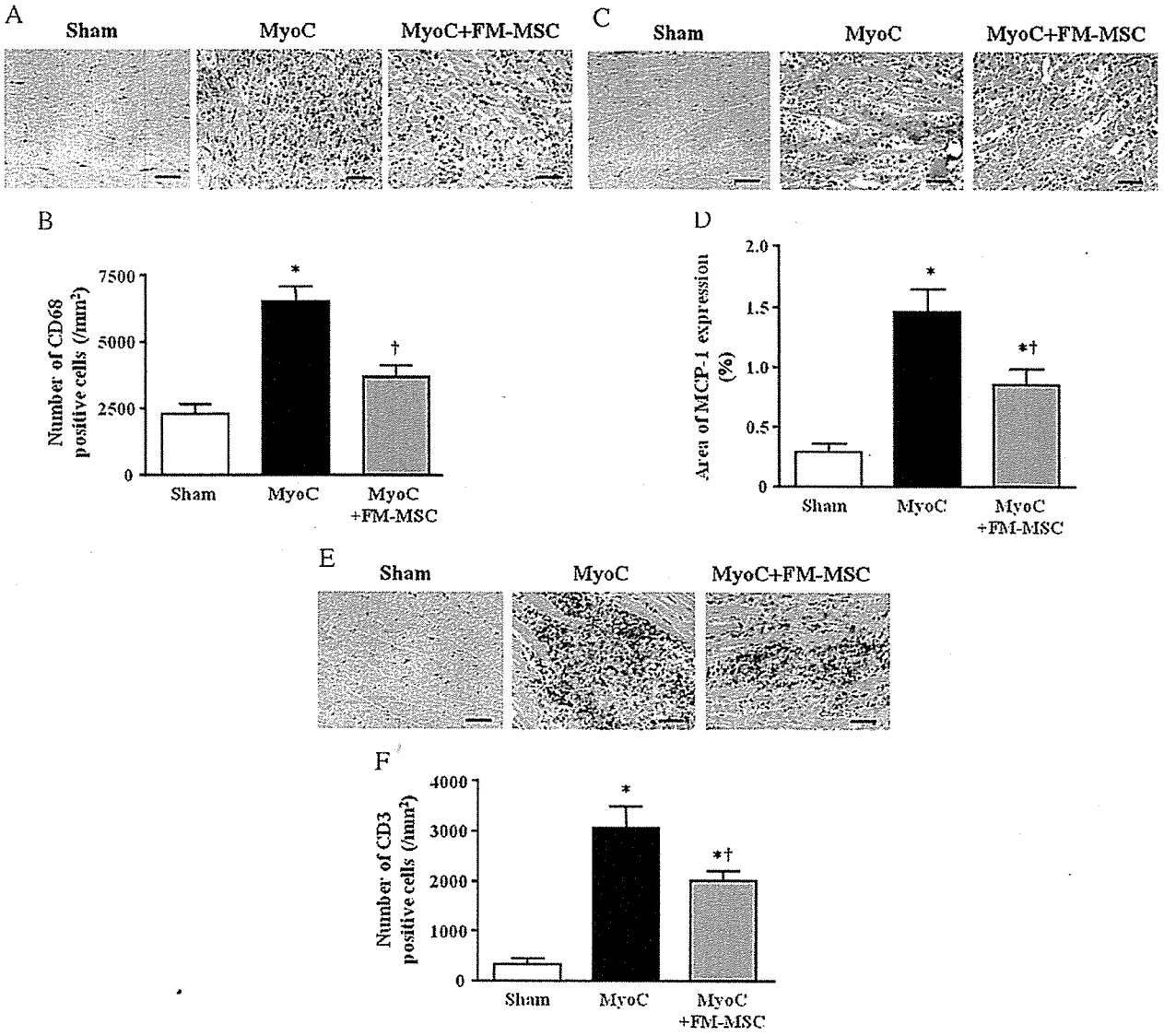

Fig. 5. Effects of administration of allogeneic FM-MSC on myocardial infiltration of inflammatory cells and MCP1 expression in acute myocarditis. (A, B) CD68-positive macrophage/monocyte infiltration was markedly lower in the MyoC + FM-MSC group than in the MyoC group. (C, D) The area of MCP1 expression was significantly smaller in the MyoC + FM-MSC group than in the MyoC group. (E, F) Significantly fewer infiltrating CD3-positive T cells were observed in the allogeneic FM-MSC group than in the MyoC group. Scale bars = 50 μm, $n = 15$ for each group. Data are expressed as mean ± SEM. $^{*}P<0.05$ vs the Sham group; $^{\dagger}P<0.05$ vs the MyoC group.

Because, the migration of activated T cells into the myocardium is considered the initial process of EAM [20]. Subsequently, large numbers of inflammatory cells, including macrophages and T cells, infiltrate the myocardium where they induce severe inflammation.

In this study, GFP-positive cells were detected in the heart tissue one day and one week after the intravenous administration of GFP-expressing FM-MSC, although only a few engrafted cells were found, even one day after injection, which is consistent with a previous work [6]. The low percentage of cells migrating to the heart is in agreement with other reports [21–23]. In several clinical applications, MSC are administered preferentially by an intravenous route [24,25]. However, limited data are available regarding the fate of systemically infused MSC. Studies in rodents suggest that a broad distribution of transplanted MSC is observed initially, followed by a limited capacity for sustained engraftment [22,26]. In addition, the number of von Willebrand factor-positive capillaries in the heart did not increase in the EAM rats given FM-MSC compared with the untreated EAM group two weeks after cell administration (data not shown), and we found no GFP-positive cells and cardiomyocyte-differentiated cells four weeks after cell administration. A recent study reported that engraftment of MSC is very low and that transplanted MSC appear to differentiate into cardiomyocytes at a very low frequency [27]. These results suggest that the angiogenic effect and the cardiomyocyte differentiation of FM-MSC in the heart are not the main effects in EAM therapy.

We have demonstrated that some of the administered GFP-positive cells were found in the lung, spleen, and liver. It is interesting that the number of GFP-positive cells in the spleen, the hub of immunoreactions of T cells, was greater at one week after administration than at one day after administration. One may speculate that the systemically engrafted allogeneic FM-MSC can exert a therapeutic effect in the treatment of myocarditis.

Recent studies have highlighted the potential immunomodulatory or anti-inflammatory effects of MSC [28,29]. MSC can suppress T-cell

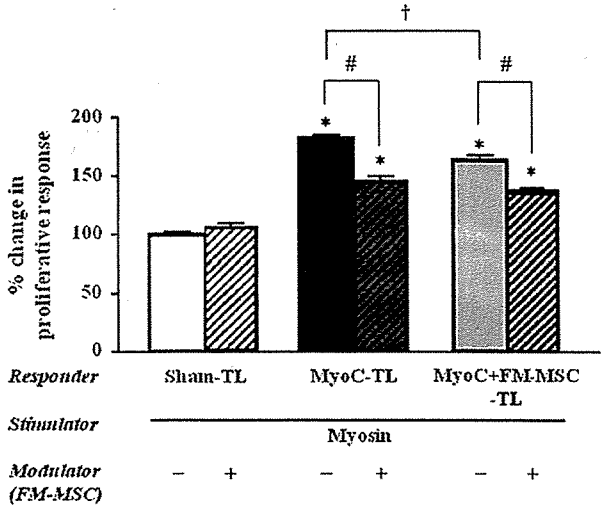

Fig. 6. Suppressive effects of T-lymphocyte activation by allogeneic FM-MSC. The T-lymphocyte proliferative response was significantly lower in the MyoC + FM-MSC group than in the MyoC group. The T-lymphocyte-activated proliferation was markedly attenuated by allogeneic FM-MSC ($n = 8$ each). Data are expressed as mean ± SEM. *$P<0.05$ vs Sham-TL; †$P<0.05$; #$P<0.05$.

activation and proliferation both in vitro and in vivo [30,31]. Recent clinical studies found that the intravenous injection of BM-MSC ameliorates acute graft-versus-host disease [13,14,32]. Interestingly, the suppression of T-cell proliferation by MSC causes no immunological restriction, insofar as similar suppressive effects were observed with cells that were either autologous or allogeneic to the responder cells [33,34]. Several independent reports have suggested multiple mechanisms by which MSC inhibit T-cell responses. Prostaglandin E2, nitric oxide, indoleamine 2,3-dioxygenase, and galectin are among the molecules postulated to be involved in the inhibition of T-cell proliferation by MSC [35–39]. Our present study demonstrated that allogeneic administration of FM-MSC significantly attenuates the infiltration of macrophages and T cells into EAM hearts. In the T-lymphocyte proliferation assay, splenic T lymphocytes derived from rats with myocarditis given allogeneic FM-MSC had a reduced activated proliferative response compared with the response of splenic T lymphocytes from untreated myocarditis rats, even two weeks after the injection of FM-MSC. In addition, activated T-lymphocyte proliferation was also suppressed by coculture with allogeneic FM-MSC in vitro. These results suggest that allogeneic FM-MSC reduce the severity of EAM by inhibiting T-cell activation and proliferation through both a direct effect and a systematic effect, and that these combined effects lead to the amelioration of impaired cardiac function.

However, GFP-positive cells could not be detected in the spleen, lung, and liver as with the heart at four weeks after administration. In this study, we administered a low number of FM-MSC to avoid pulmonary embolism. More effective results might be obtained with administration of more cells or multiple administrations of FM-MSC in EAM. One study reported that transplantation of allogeneic BM-MSC into a rat model of myocardial infarction improved cardiac functions at four weeks after transplantation, although this benefit was transient [40]. Currently, we are extending our observation up to three months in EAM. Although the results are an inadequate number to be presented, improvement of cardiac function appears to be maintained in the intravenous allogeneic FM-MSC administration group compared with the untreated EAM group.

In conclusion, this study showed that the intravenous allogeneic administration of FM-MSC ameliorated cardiac dysfunction in a rat model of acute myocarditis. These beneficial effects may be mainly attributable to the suppression of T-lymphocyte activation rather than to angiogenesis and cardiomyocyte differentiation of the administered allogeneic FM-MSC. Although further experiments are needed to apply the current results to human cardiomyoplasty, the allogeneic administration of FM-MSC may provide a new therapeutic strategy for the treatment of severe acute myocarditis.

Acknowledgments

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Research paper

Ulcerated plaques in the aortic arch contribute to symptomatic multiple brain infarction

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ABSTRACT

Background The configuration of aortic plaque appears to be as important as its thickness when it acts as an embolic source to the brain. The frequency and clinical predictors of ulcerated plaque at the aortic arch identified using transoesophageal echocardiography (TOE) in patients with stroke were determined, and the association between the ulcer and recent ischaemic stroke, particularly multiple brain infarcts, which appear to indicate embolic stroke, was examined.

Methods Aortic and cardiac embolic sources were evaluated using TOE in 360 patients with fresh ischaemic stroke proven by diffusion-weighted MRI, including 210 patients with a single infarct and 150 with multiple infarcts, as well as in 101 non-stroke control patients. An ulcer was defined as a crater on the plaque ≥2.0 mm in depth and width.

Results An ulcerated plaque was identified in 10.6% of patients with stroke versus 2.0% of non-stroke patients, showing a 5.11-fold higher frequency in patients with stroke (95% CI 1.51 to 31.96) after adjustment for age and sex. After multivariate adjustment for clinical and ultrasonographic features, multiple-infarct patients had a 7.61-fold higher risk (95% CI 1.99 to 50.43) of having an ulcer than control patients and a 3.32-fold higher risk (95% CI 1.61 to 7.18) of having an ulcer than single-infarct patients. Diabetes mellitus and drinking habit were independently related to the presence of ulcerated plaque in patients with stroke.

Conclusions Ulcerated plaque in the aortic arch was associated with the development of ischaemic stroke, especially multiple brain infarcts, probably involving, at least in part, an embolic mechanism.

INTRODUCTION

Compared with atrial fibrillation and severe carotid artery stenosis, the aortic arch has received limited attention as an embolic source to the brain.[1 2] However, several autopsy studies and transoesophageal echocardiography (TOE) studies reported a strong association between aortic atheroma and the development of stroke.[3–14] In most TOE studies, plaque thickness, usually ≥4.0 mm, was regarded as an important risk factor for stroke. In contrast, autopsy studies stressed that ulcerated plaques in the aortic arch had a relationship with stroke.[3 5] Thus, the plaque configuration seems to be as important as the plaque thickness when aortogenic embolism is assessed.

Ulceration is a typical finding of advanced atherosclerosis and represents fertile ground for potential thrombosis and consequent embolic events. Ulcerated plaques in the aortic arch have been reported to be more common in patients with cryptogenic stroke than in control patients.[15 16] However, such plaques may be innocent bystanders of atherothrombotic stroke. To prove that aortic plaques can be sources of emboli to the brain, Ueno *et al*[17] studied the relationship between TOE findings and multiple brain infarcts, and they concluded that mobile aortic plaques contribute to embolic stroke. Multiple hyperintense lesions on diffusion-weighted images (DWIs) are reported to strongly indicate an embolic stroke mechanism.[18 19]

The first goal of this study was to determine the frequency of ulcerated plaque at the aortic arch detected by TOE in patients with stroke and to examine whether ulcerated plaque is more likely in patients with stroke than in non-stroke patients after multivariate adjustment. In particular, the relationship between ulcerated plaque and recent multiple brain infarcts identified on DWI, which appear to indicate embolic stroke, was examined. The second goal of this study was to clarify the clinical background of patients with stroke having aortic ulcers and to determine the clinical predictors of aortic ulcers.

METHODS

From January 2005 to January 2007, 960 patients were admitted to our stroke centre within 7 days after onset of acute ischaemic stroke. Of these, 374 (39.0%) consecutive patients underwent TOE for evaluation of the aortic arch and the heart. In 14 of them, MRI was contraindicated mainly due to pacemaker implantation; the other 360 underwent brain MRI to identify fresh brain infarcts. These 360 patients were enrolled in this study (248 men, 70±11 years old). The remaining 586 patients with stroke did not undergo TOE because the vascular neurologists in charge did not think that TOE was needed for patient management. In our stroke centre, TOE is considered to be semi-invasive and is principally used in patients with stroke of undetermined aetiology, those with possible paradoxical embolism and those with cardioembolic stroke for whom detailed evaluation of intracardiac thrombus is required. As a control group, 101 consecutive patients receiving TOE and MRI during the same period who did not have previous ischaemic stroke, transient ischaemic attack (TIA) or silent brain infarcts on MRI were also enrolled (58 men, 65±13 years old). Of these 101 patients, 60 were initially suspected of having developed stroke or

TIA, but their final diagnosis was not stroke or TIA; the remaining 41 neurological patients who did not have stroke underwent TOE mainly for the evaluation of atrial fibrillation or systemic atherosclerosis.

The following underlying risk factors and comorbidities were examined: age, sex, hypertension (blood pressure ≥140/90 mm Hg before stroke onset or history of antihypertensive medication), diabetes mellitus (fasting blood glucose ≥7.0 mmol/l, positive 75 g oral glucose tolerance test or history of antidiabetic medication), hypercholesterolaemia (serum total cholesterol ≥5.7 mmol/l or history of antihypercholesterolemic medication), coronary heart disease, peripheral artery disease, aortic aneurysm, smoking habit (previous and current) and drinking habit (≥2 drinks per day). The use of antiplatelet agents or warfarin for ≥6 months before stroke onset was also assessed.

To make a diagnosis of ischaemic stroke, identification of culprit infarcts on DWI of MRI, in addition to the episode of neurological dysfunction, was required. Multiplicity of fresh infarcts was also assessed using DWI. Multiple infarcts were defined as more than one high-intensity area in a slice or high-intensity areas in more than one slice that were not a series of a single lesion. For examination of cephalocervical artery lesions, MR angiography and duplex carotid ultrasonography were performed in all patients. Arterial stenosis was identified as the presence of a stenosis ≥50% in diameter. For screening of cardiac embolic sources, 24 h electrocardiography and transthoracic echocardiography, as well as TOE, were performed in all patients. High-risk emboligenic heart diseases, including atrial fibrillation, were assessed based on the criteria of the Trial of Org 10172 in Acute Stroke Treatment (TOAST) study.[20]

To evaluate aortic lesions, a commercially available, real-time, two-dimensional echocardiography system (SSD-2200, ALOKA, Tokyo, Japan) equipped with a 5.0 MHz phased array omniplane transoesophageal transducer was used. The thoracic aortic arch was observed with both transverse and sagittal views, and, if needed, with other appropriate views, as described in our previous study.[6] Possible aortogenic embolic sources to the brain included: a large atheromatous plaque, defined by focal increases in intima-media thickness (IMT) ≥4.0 mm[8]; a plaque at the origin of the left subclavian artery with IMT ≥2.0 mm; spontaneous echo contrast (SEC); and ulcerated plaque. An ulcer was defined as a crater on the plaque ≥2.0 mm in depth and width, as in previous studies (figure 1).[10 15 16 21] In addition, TOE was used to detect possible cardiac embolic sources, including intracardiac thrombus, an SEC in the left atrium or atrial appendage, a mitral strand, an atrial septal aneurysm and a right-to-left shunt documented on a contrast-enhanced technique.[22] Each finding on TOE was assessed by trained vascular neurologists and, sometimes, by additional trained cardiologists.

Statistical analysis was performed using the JMP 7 software package (SAS Institute, Cary, North Carolina). A multivariate logistic regression analysis was performed to identify associations between underlying clinical characteristics and TOE findings in patients with stroke versus non-stroke control patients or patients having multiple infarcts on DWI versus patients having a single infarct. These analyses were done with adjustment for sex and age (model 1) and with adjustment for sex, age and variables chosen using a stepwise selection method (model 2). The same type of multivariate logistic regression analysis was performed to identify clinical predictors for the presence of ulcerated plaque in the aortic arch in patients with stroke. A value of $p<0.05$ was considered statistically significant, and that of $p<0.10$ was considered marginally significant.

RESULTS

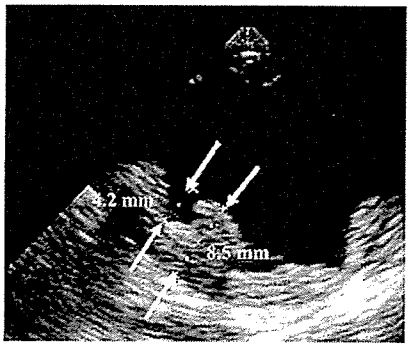
Frequency of ulcerated plaque

An ulcerated plaque on the aortic arch was identified in two (2.0%) of 101 non-stroke control patients and in 38 (10.6%) of 360 patients with stroke (figure 2). The depth of the ulcer was 2.1 mm and 3.0 mm in two control patients, and ranged from 2.1 to 9.1 mm (median 3.3 mm) in the 38 patients with stroke. Of the 360 patients with stroke, 150 patients (41.7%) had multiple fresh infarcts proven on DWI, and 210 had a single fresh infarct. An ulcerated plaque was identified in 12 patients with a single infarct (5.7%) and 26 patients with multiple infarcts (17.3%). The depth of the ulcer ranged from 2.4 to 5.1 mm (median 3.0 mm, IQR 2.5 to 4.0 mm) in patients with a single infarct, and from 2.1 to 9.1 mm (median 3.4 mm, IQR 2.7 to 4.4 mm) in patients with multiple infarcts.

Association of ulcerated plaque with stroke versus non-stroke controls

The underlying characteristics and TOE findings of non-stroke control patients and patients with stroke are listed in table 1. After adjustment for age and sex (model 1), patients with stroke had a 5.11-fold higher risk (95% CI 1.51 to 31.96, p=0.027) of having an ulcerated plaque in the aortic arch compared with control patients. In addition, coronary heart disease (p=0.019), cephalocervical arterial stenosis (p=0.008) and SEC in the left atrium (p=0.007) were more common in patients with stroke than control patients after adjustment for age and sex.

The multivariate-adjusted ORs and 95% CIs (model 2) for underlying characteristics and TOE findings in patients with stroke, using the values in non-stroke patients as a reference, are listed in table 2. An ulcerated plaque tended to be more common (OR 4.21, 95% CI 1.19 to 26.93, p=0.057), while advanced age (p=0.002), coronary heart disease (p=0.029), cephalocervical arterial stenosis (p=0.041) and SEC in the left atrium (p=0.008)



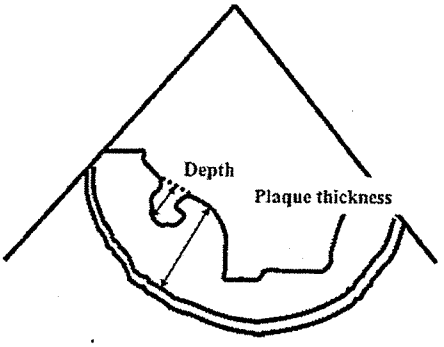


Figure 1 Representative ultrasonogram of an ulcer, 4.2 mm in depth, on a large atheromatous plaque, 8.5 mm in thickness, in the aortic arch.

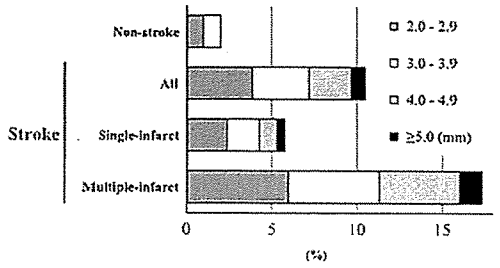


Figure 2 Frequency of ulcerated plaque categorised by maximum ulcer depth.

were significantly more common in patients with stroke than in control patients. In contrast, the frequency of a large arch plaque (≥4.0 mm) was no different between patients with stroke and control patients on multivariate analysis.

Association of ulcerated plaque with multiple brain infarcts

The underlying characteristics and TOE findings in patients with stroke with a single infarct and in those with multiple infarcts are also listed in table 1. Compared with non-stroke control patients, multiple-infarct patients more commonly had cephalocervical arterial stenosis (p=0.002), ulcerated plaque in the aortic arch (p=0.003), large arch plaque (p=0.007) and SEC in the left atrium (p=0.017) after adjustment for age and sex (model 1). Compared with single-infarct patients, multiple-infarct patients more commonly had an ulcerated plaque (p=0.001) and a large arch plaque (p=0.047) after adjustment for age and sex.

The multivariate-adjusted ORs and 95% CIs (model 2) for the underlying characteristics and TOE findings in patients with multiple infarcts, using those in non-stroke patients and in single-infarct patients as the reference, are listed in table 3. The multiple-infarct patients had a 7.61-fold higher risk (95% CI 1.99 to 50.43, p=0.010) of having an ulcerated plaque in the arch compared with control patients. In addition, advanced age (p=0.001) and cephalocervical arterial stenosis (p=0.032) were more common in multiple-infarct patients than in control patients. Only an ulcerated plaque was independently associated with multiple brain infarcts compared with a single infarct (OR 3.32, 95% CI 1.61 to 7.18, p=0.002).

Clinical predictors for an ulcerated plaque in patients with stroke

Table 4 shows the underlying risk factors and comorbidities of patients with stroke with and without an ulcerated plaque in the aortic arch. After multivariate adjustment (model 2), diabetes mellitus (OR 3.26, 95% CI 1.56 to 6.86, p=0.002) and drinking habit (OR 4.22, 95% CI 1.42 to 12.17, p=0.008) were independently related to the presence of ulcerated plaque.

DISCUSSION

In the present study, the association between ulcerated plaque in the aortic arch and recent development of presumed embolic stroke was determined. The first major finding was that patients with a recent stroke more commonly had a TOE-identified aortic ulcer than non-stroke control patients after adjustment for age and sex, and they tended to have it after multivariate adjustment for clinical and ultrasonographic features. The

Table 1 Underlying characteristics and transoesophageal echocardiography (TOE) findings

Variables	Non-stroke N=101	Stroke All, N=360	Single infarct, N=210	Multiple infarcts, N=150
Age (years)	65±13	70±11*	69±11*	71±10*
Male sex	58 (57)	248 (69)*	144 (69)*	104 (69)*
Underlying characteristics				
Hypertension	62 (61)	270 (75)	155 (74)	115 (77)
Diabetes mellitus	16 (16)	100 (28)	58 (28)	42 (28)
Hypercholesterolaemia	35 (35)	153 (43)	87 (41)	66 (44)
Coronary heart disease	8 (8)	80 (22)*	50 (24)*	30 (20)
Peripheral artery disease	2 (2)	15 (4)	9 (4)	6 (4)
Aortic aneurysm	3 (3)	11 (3)	6 (3)	5 (3)
Smoking habit	43 (43)	198 (55)	116 (55)	82 (55)
Drinking habit	15 (15)	39 (11)	20 (10)	19 (13)
Antiplatelet pretreatment	19 (19)	82 (23)	52 (25)	30 (20)
Warfarin pretreatment	11 (11)	42 (12)	26 (12)	16 (11)
Cephalocervical arterial stenosis	23 (23)	142 (39)*	74 (35)*	68 (45)*
High-risk emboligenic heart disease	22 (22)	125 (35)	77 (37)	48 (32)
(Atrial fibrillation)	20 (20)	118 (33)	70 (33)	48 (32)
TOE findings of the aorta				
Ulcerated plaque	2 (2)	38 (11)*	12 (6)	26 (17)* †
Large plaque (≥4 mm)	16 (16)	114 (32)	56 (27)	58 (39)* †
Plaque on left subclavian artery	19 (19)	82 (23)	50 (24)	32 (21)
SEC in the aortic arch	4 (4)	38 (11)	19 (9)	19 (13)
TOE findings of the heart				
Intracardiac thrombus	3 (3)	18 (5)	11 (5)	7 (5)
SEC in the left atrium	9 (9)	88 (24)*	51 (24)*	37 (25)*
Mitral strand	17 (17)	56 (16)	28 (13)	28 (19)
Right-to-left shunt	18 (18)	73 (21)	43 (20)	30 (20)
Atrial septal aneurysm	2 (2)	8 (2)	6 (3)	2 (1)

Values are number (percentage) or mean ± SD; *p<0.05 versus 'Non-stroke' and †p<0.05 versus 'Single infarct' after adjustment for age and sex (model 1); SEC, spontaneous echo contrast.

Table 2 Multivariate-adjusted OR and 95% CI for underlying characteristics and transoesophageal echocardiography findings in patients with stroke versus non-stroke patients

Variables	OR	95% CI	p Value
Age, per 1-year increase	1.03	1.01 to 1.05	0.002
Male sex	1.39	0.79 to 2.48	0.256
Coronary heart disease	2.43	1.15 to 5.80	0.029
Aortic aneurysm	0.41	0.10 to 2.05	0.225
Smoking habit	1.66	0.94 to 2.94	0.080
Drinking habit	0.56	0.27 to 1.21	0.132
Cephalocervical arterial stenosis	1.75	1.03 to 3.04	0.041
Ulcerated plaque	4.21	1.19 to 26.93	0.057
Spontaneous echo contrast in the left atrium	2.80	1.36 to 6.39	0.008

Age, sex and variables that were chosen by a stepwise selection method are listed (model 2).

second major finding was that patients with stroke with multiple infarcts, indicating an embolic aetiology, more commonly had an aortic ulcer than non-stroke patients and single-infarct stroke patients after multivariate adjustment. The third major finding was that diabetes mellitus and drinking habit were independently related to the presence of aortic ulcer in patients with stroke.

Some studies have examined the association between ulcerated plaque and stroke development. Stone *et al*[15] found that the frequency of aortic arch ulcer was much higher in 23 patients with cryptogenic ischaemic stroke (39%) than in 26 patients with known-cause strokes (8%) and 57 non-stroke control patients (7%). Di Tullio *et al*[16] demonstrated that ulcerated or mobile plaque in the aortic arch was more frequent in patients with cryptogenic stroke aged ≥60 years than same-aged control subjects (OR 3.4, 95% CI 1.1 to 11.2). Recently, they showed that, compared with no plaques in the arch, ulcerated or mobile plaque ≥4.0 mm was associated with a 3.3-fold increase (95% CI 1.4 to 8.2) in the risk of ischaemic stroke after adjustment for other stroke risk factors.[21]

The frequency of aortic ulcer in the present stroke (10.6%) and non-stroke patients (2.0%) was smaller than in the previous report on TOE[15] and in pathological studies.[3 5] In the above-mentioned report by Stone *et al*,[15] 39% of patients with cryptogenic stroke had a TOE-proven aortic ulcer. On autopsy, 26% of cerebrovascular patients had an ulcer.[3] A large difference in the frequency between the previous studies and the present study may result from less severe atherosclerosis of the large arteries in the Japanese population than in the Western population, partly because elderly Japanese have a relatively low-cholesterol diet. White subjects were reported to have more severe aortic lesions than black or Hispanic subjects on TOE.[16 23] However, the adjusted odds ratio for the presence of ulcer in patients with stroke versus control patients was similar in the present study to that in the previous French pathological study (4.0).[3]

To be able to conclude that aortic ulcer is a definite cause of stroke, it is reasonable to assess the presence of ulcer in patients with cryptogenic stroke who do not have other stroke causes. However, one needs to determine how minor cardiac abnormalities and aortic findings identified only on TOE should be handled. For example, aortic ulcers generally lie on large plaques ≥4.0 mm in thickness.[9] If a plaque ≥4.0 mm is considered a known cause of stroke, and patients with such a plaque are excluded from patients with cryptogenic stroke, most patients with aortic ulcer are not cryptogenic and are excluded from the assessment. As an alternative, assessment of multiple-infarct patients with adjustment for other stroke causes seems to be logical. Multiple infarcts are typical of embolic stroke.[18 19] The present high OR for the presence of aortic ulcer in multiple-infarct patients versus non-stroke or single-infarct patients after adjustment for several other stroke causes indicates that the ulcer is not necessarily an innocent bystander in patients with stroke but is a potential embolic source. In the above-mentioned French pathological study, 14 of 17 patients who did not have apparent stroke causes other than the aortic ulcer had multiple brain infarcts.[3]

In general, the age of the plaque and its longstanding history are reflected by the thickness. In contrast, ulceration on the plaque may develop by the plaque rupture immediately before stroke onset or may be a longstanding hotbed of platelet aggregation. A pathological study on aortogenic embolic stroke from our institute proved the existence of atheromatous emboli composed mostly of cholesterol crystals in culprit cerebral arteries,[4] suggesting recent plaque rupture. In some of our patients with recurrent embolic stroke, the aortic ulcer did not change its shape on TOE between the initial stroke and the recurrent stroke. In a few patients, the ulcer and mobile components of the plaque changed the shape after the recurrent event. A prospective observational study with repeated TOE examinations is required to determine the association between embolic events and the appearance of a new ulcerated lesion.

Table 3 Association of underlying characteristics and transoesophageal echocardiography findings with multiple infarcts after multivariate adjustment

	Versus non-stroke			Versus single infarct		
Variables	OR	95% CI	p Value	OR	95% CI	p Value
Age, per 1-year increase	1.05	1.02 to 1.08	0.001	1.02	1.00 to 1.05	0.060
Male sex	1.59	0.87 to 2.94	0.136	1.13	0.70 to 1.84	0.617
Coronary heart disease	2.44	0.94 to 6.98	0.078	0.64	0.37 to 1.10	0.113
Antiplatelet pretreatment	0.56	0.24 to 1.27	0.173			
Cephalocervical arterial stenosis	2.02	1.07 to 3.88	0.032	1.38	0.89 to 2.16	0.153
High-risk emboligenic heart disease				0.75	0.47 to 1.20	0.231
Ulcerated plaque	7.61	1.99 to 50.43	0.010	3.32	1.61 to 7.18	0.002
Large plaque (≥4 mm)	1.96	0.95 to 4.17	0.073			
Plaque on left subclavian artery	0.55	0.26 to 1.19	0.130			
SEC in the aortic arch	2.31	0.68 to 9.40	0.201			
SEC in the left atrium	2.03	0.84 to 5.21	0.126			
Mitral strand				1.45	0.70 to 2.62	0.218

Age, sex and variables that were chosen by a stepwise selection method are listed (model 2).
SEC, spontaneous echo contrast.

Table 4 Underlying characteristics of patients with stroke with and without ulcerated plaque

Variables	Without ulcer, n=322	With ulcer, n=38	'With ulcer' versus 'Without ulcer'*		
			OR	95% CI	p Value
Age (years)	70±11	72±10	1.03	0.99 to 1.08	0.133
Male sex	221 (69)	27 (71)	0.79	0.34 to 1.90	0.594
Diabetes mellitus	79 (25)	21 (55)	3.26	1.56 to 6.86	0.002
Peripheral artery disease	11 (3)	4 (11)	2.99	0.71 to 10.63	0.105
Aortic aneurysm	8 (2)	3 (8)	2.97	0.52 to 13.11	0.173
Drinking habit	31 (10)	8 (21)	4.22	1.42 to 12.17	0.008
Warfarin pretreatment	40 (12)	2 (5)	0.34	0.05 to 1.26	0.163
Cephalocervical arterial stenosis	120 (37)	22 (58)	1.98	0.91 to 4.17	0.065

Values are number (percentage) or mean ± SD.
Age, sex and variables that were chosen by a stepwise selection method are listed (model 2).
*Multivariate-adjusted for all listed variables.

Of the two risk factors that were independently associated with aortic ulcer in the present cohort, diabetes mellitus was reported to be associated with aortic atherosclerosis in some studies,[24–26] but the association disappeared after multivariate adjustment.[27] Alcohol intake has not been found to be associated with calcified aortic atherosclerosis.[28 29] Considering the conflicting effect of alcohol on vascular diseases, which depends on the amount and the kind of drinking,[30] the effect of alcohol on ulcerated plaque in the present study is inconclusive. Among various risk factors, age is the strongest and universally accepted predictor of aortic atherosclerosis,[2] and it is expected to be a predictor of aortic ulcer. The lack of a positive association between age and ulcer in our patients with stroke may be a methodological limitation because the patients' age range was not wide.

Aspirin seems to be a common choice for the treatment of patients with aortic atheromatous plaques.[5 8 9 15] Some studies reported that, compared with patients with aortic atheroma not treated with oral anticoagulants, patients treated with oral anticoagulants had a better outcome.[31–33] A recent study reported the association of large aortic plaques with an elevation in the level of prothrombin fragment F1.2, an indicator of thrombin generation.[21] On the other hand, anticoagulation may enhance plaque vulnerability and cause cholesterol embolism to the kidneys and peripheral arteries.[34 35] Thus, there is no consensus on prevention strategies for stroke in patients with aortic plaques. The Aortic Arch Related Cerebral Hazard (ARCH) study, a randomised controlled study comparing the efficacy of warfarin with that of aspirin plus clopidogrel in patients with arch atheroma and stroke or peripheral embolism is ongoing.[36] Although antiplatelet agents and warfarin were not associated with the presence of aortic ulcer after multivariate adjustment in this analysis, a study with a more sophisticated design is needed to clarify the association.

The limitations of the present study include the cross-sectional study design that involved performing TOE after the development of stroke; this may limit the analyses. A prospective case–control study to analyse the future development of stroke is required to accurately determine the contribution of aortic ulcer to stroke. The second limitation was that not all of the stroke inpatients underwent TOE, which resulted in selection bias. The third limitation was that non-stroke control patients were much fewer than patients with stroke, and the sex and age of control patients did not match those of patients with stroke; these affected the results of the statistical analysis. In particular, a small number of non-stroke patients having ulcerated plaques (2%) might cause the very wide 95% CI (1.99 to 50.43) in the comparison between non-stroke patients and multiple-infarct patients. Since we required strict MRI evidence for absence of brain infarcts in control patients, we could not include many patients as controls. The fourth limitation was that aortic ulcers located at the ascending aorta might be underdiagnosed, though they cause embolism to the brain, since a large part of the ascending aorta is a blind spot for TOE. The fifth limitation was that the contribution of aortic ulcers as a cause of embolism to a single brain territory was underestimated. Finally, artery-to-artery embolism is a likely cause of multiple infarcts in the same vascular territories. To exclude influences of other embolic sources than aortic lesions, we performed statistical analyses using adjustment for cephalocervical arterial stenosis and high-risk emboligenic heart disease.

In conclusion, this is the first study to identify a strong association between ulcerated plaque in the aortic arch and recent multiple brain infarctions, even after adjustment for other stroke causes. This strong association suggests that aortic ulcers cause embolism to multiple brain territories. The characteristic configuration of the aortic plaque appears to be as important as plaque thickness.

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Competing interests None.

Ethics approval Ethics approval was provided by the local ethics committee. in National Cerebral and Cardiovascular Center

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NOTE

Association of plasma B-type natriuretic peptide levels with obesity in a general urban Japanese population: the Suita study

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Abstract. The inverse association between plasma B-type natriuretic peptide (BNP) levels and body mass index (BMI) has been reported in Western populations. Here we analyzed the relationship between plasma BNP and obesity in a general urban Japanese population. We recruited 1,759 subjects without atrial fibrillation or history of ischemic heart disease aged 38-95 years (mean age ± standard deviation 64.5 ± 10.9 years, 56.1% women, mean BMI 22.8 ± 3.1 kg/m^2) from the participants in the Suita Study between August 2002 and December 2003. In multivariable regression analyses adjusted for age, systolic blood pressure, pulse rate, serum creatinine, left ventricular hypertrophy in ECG, the inverse relationships between BNP levels and BMI (kg/m^2) was found in both sexes (both $p<0.001$). Multivariable-adjusted mean plasma BNP levels in the group of BMI<18.5, 18.5≦BMI<22, 22≦BMI<25, and 25≦BMI were 23.4, 17.9, 14.0 and 13.0 pg/mL, respectively (trend $p<0.001$). The negative association of body fat (percentage and mass), skin fold thickness, or waist circumference with BNP levels was observed in both sexes ($p<0.01$). Among the obesity indices, body fat mass is most tightly associated with BNP. In conclusion, plasma BNP was inversely associated with obesity-related markers such as body fat mass, skinfold thickness and waist circumferences after adjusted for relevant covariates in a Japanese population.

Key words: BNP, BMI, Body fat mass, Japanese

B-TYPE natriuretic peptide (BNP) is a cardiac hormone, synthesized in, processed in and secreted from heart [1]. The secretion of BNP is stimulated in heart failure along with its severity. Plasma BNP is clinically utilized to diagnose the existence or the severity of the cardiac failure [2]. BNP levels are affected by demographic variables such as age, gender, and clinical characteristics such as hypertension [3, 4], atrial fibrillation [5], and renal function [6].

Several recent studies have suggested that obesity, as indexed elevated body mass index (BMI), also affects BNP levels, with lower circulating levels in those with higher BMI in subjects with and without heart failure [7-12]. In addition, the Dallas Heart Study revealed that BNP is closely associated with lean mass than with fat mass [8]. However, these studies were mostly conducted in Western countries, where BMI is much higher than in other parts of the world. It is not unclear whether this relationship could apply in a general Japanese population whose BMI levels are lower than in Western countries [13].

Therefore, the aim of the present study is to evaluate the association between BMI and BNP levels in a general urban Japanese population. To further elucidate the mechanisms of the relationship between obesity and BNP levels, we examine the relationship between BNP levels and various obesity related factors such as lean body mass, body fat mass, skin fold thickness, and waist circumferences.

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Methods

Study Sample

The Suita Study [14-16], an epidemiological study of cerebrovascular and cardiovascular disease, was based on a random sampling of 15,200 (30–79 years of age at enrollment) Japanese residents of Suita. They were all invited, by letter, to attend regular cycles of follow-up examination (every 2 years). Subjects were recruited into the Suita Study between August 2003 and December 2004 in this study (n=2,007). Subjects with chronic atrial fibrillation at time of referral (n=40) and history of ischemic heart disease (n= 97) were excluded. After applying this exclusion, 1,759 individuals were included in this analysis. The study design was approved by the institutional review board of the National Cardiovascular Center. Informed consent was obtained from all subjects.

Routine physical examination, 12-lead surface ECG, several blood chemical variables and plasma BNP measurements were performed. A physician or nurse interviewed each patient personal history of cardiovascular disease, including angina pectoris and/or myocardial infarction. Blood pressure was measured after at least 5 minutes of rest in a sitting position. Systolic and diastolic blood pressures (SBP and DBP) were the means of two measurements by well-trained doctors (recorded at least 1 min apart) [16]. BMI was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured in a standing position at the umbilical level by well-trained technicians [15]. Lean body mass and body fat mass were calculated by the bioelectrical impedance analyzer [17]. Brachial triceps and subscapular skin fold thickness was measured using keys calipers by trained physician epidemiologists with standard methods.

Measurement of BNP

Blood sample was collected into tubes containing EDTA. Plasma BNP was measured by validated and commercially available immunoassay kit (Shionogi, Osaka, Japan). The measurable range of the BNP assay is 4.0 to 2000 pg/mL. Average intra- and inter-assay coefficients of variation were 3.7% and 4.5%, respectively.

Statistical Analyses

Continuous data are presented as means ± standard deviations (SDs) for normally distributed variables and as medians (interquartile range) in case of skewed distribution. Categorical data are presented as numbers and percentage. Comparison of clinical characteristics between patients each BMI category were performed using Kruskal-Wallis test for continuous data and χ^2 test for categorical data. Variables with skewed distributions underwent logarithmic transformation to create normal distributions. The value less than the lower detection limit of the BNP assay (BNP < 4.0 pg/mL) were found 9.0% of all subjects. For analyses examining continuous BNP levels, we treated lower detection limit of the BNP assay for 4.0 pg/mL and performed multivariable linear regression with log-transformed BNP as the dependent variables. Covariates examined for inclusion in the multivariable models were age, sex, systolic blood pressure, pulse rate, serum creatinine, and left ventricular hypertrophy (LVH) in ECG. Sex-specific regression analyses were also performed. In additional models, we replaced the continuous BMI variable with BMI categories (BMI<18.5, 18.5 ≦ BMI< 22, 22 ≦ BMI < 25, and 25 kg/m^2 ≦ BMI). The results of the multivariable analyses were also used to examine the relations of BMI category to adjusted plasma BNP levels. Since models used log-transformed dependent variables, we exponentiated the β coefficient for BMI to characterize its multiplicative effect on absolute plasma BNP levels. Because of the skewed nature of the BNP distributions and potential violations of assumptions inherent in the least-squares model, we used multivariable logistic regression analyses to analyze correlations of normal plasma BNP levels (BNP<18.4 pg/mL). We estimated odds ratios for having normal BNP levels according to BMI category, with lowest BMI individuals as the referent group. Odds ratios were adjusted for the same covariates used in the linear models.

All data were analyzed with the JMP version 6.0 (SAS Corporation, Cary, NC, USA) statistical software package.

Results

Baseline Characteristics

The clinical characteristics of study population (mean ± SDs age 64.5 ± 10.9 years, mean BMI 22.8 ± 3.1 kg/m^2, 56.1% women) stratified by BMI category are listed in Table 1. Increasing BMI was associated with an increased likelihood of being men; higher systolic blood pressure, and lower BNP levels.

Table 1 Baseline characteristics stratified by BMI: the Suita Study.

	BMI<18.5 (n=139)	18.5 ≦ BMI<22 (n=590)	22 ≦ BMI<25 (n=642)	25 ≦ BMI (n=388)	*p* value
Age, y	65.6 ± 11.5	64.2 ± 11.5	64.7 ± 10.6	64.0 ± 10.3	0.471
Men, %	28.1	35.3	50.5	51.8	<0.001
Smoking (ever), %	88.5	82.5	72.0	74.7	<0.001
Alcohol (ever), %	97.1	97.5	96.3	97.2	0.658
Hypertension, %	23.7	23.4	37.1	48.7	<0.001
Diabetes Mellitus, %	4.3	4.1	8.9	13.7	<0.001
Dyslipidemia, %	11.5	21.7	26.3	30.7	<0.001
Left ventricular hypertrophy, %	7.2	10.0	11.4	7.2	0.119
Height, cm	157.2 ± 8.0	157.3 ± 8.0	159.0 ± 9.3	158.7 ± 8.9	0.003
Weight, kg	43.2 ± 5.1	50.9 ± 5.8	59.4 ± 7.3	68.5 ± 9.4	<0.001
Skinfold thickness, mm	21.1 ± 6.8	26.5 ± 7.5	31.3 ± 8.9	37.9 ± 11.4	<0.001
Body fat mass, kg	7.9 ± 1.8	12.3 ± 2.3	16.1 ± 2.6	22.1 ± 4.5	<0.001
Lean body mass, kg	35.4 ± 4.9	38.6 ± 5.9	43.3 ± 8.0	46.4 ± 8.9	<0.001
Waist circumference, cm	71.6 ± 5.0	78.9 ± 5.3	86.5 ± 5.1	94.6 ± 6.6	<0.001
Systolic blood pressure, mmHg	117 ± 21	120 ± 18	125 ± 19	131 ± 19	<0.001
Diastolic blood pressure, mmHg	71 ± 11	74 ± 10	76 ± 10	80 ± 10	<0.001
Pulse rate, bpm	68 ± 10	67 ± 10	67 ± 9	67 ± 10	0.252
Serum creatinine, mg/dL	0.69 ± 0.21	0.68 ± 0.17	0.71 ± 0.16	0.72 ± 0.23	<0.001
Fasting plasma glucose, mg/dL	93 ± 18	94 ± 15	100 ± 21	105 ± 22	<0.001
HbA1c, %	5.4 ± 0.8	5.3 ± 0.5	5.5 ± 0.7	5.6 ± 0.8	<0.001
Triglyceride, mg/dL	80 ± 55	92 ± 55	109 ± 72	133 ± 86	0.722
Total cholesterol, mg/dL	208 ± 31	210 ± 32	211 ± 33	211 ± 32	<0.001
HDL cholesterol, mg/dL	73 ± 17	64 ± 15	59 ± 14	54 ± 13	<0.001
BNP, pg/mL	23.2 (14.7, 41.8)	18.6 (9.0, 32.6)	14.5 (7.5, 26.0)	13.2 (6.4, 24.2)	<0.001

Results presented are mean ± SD for continuous variables or percentage for categorical variables.
BNP levels are presented as median (25th, 75th percentile).
Comparison of clinical characteristics between patients each BMI category were performed using Kruskal-Wallis test for continuous data and χ^2 test for categorical data.

When BNP level was categorized by quartile (data not shown), increasing BNP levels was associated with an increased likelihood of being women and older; lower BMI, and pulse rate; an increased likelihood of having LVH; and higher systolic blood pressure.

Association between BMI and BNP Levels

Results of multivariable regression models are shown in Table 2. After adjustment for age, systolic blood pressure, pulse rate, serum creatinine, and LVH, BMI was inversely associated with plasma BNP levels, with 5% decrease associated with each 1 unit increase in BMI in both sexes (p<0.001 for both). There was also a progressive decrease in plasma BNP levels with increasing BMI category. In men, highest BMI group (BMI ≧ 25) had 29% lower plasma BNP levels compared with lowest BMI group (BMI < 18.5) (p<0.001). In women, highest BMI group (BMI ≧ 25) had 23% lower plasma BNP levels compared with lowest BMI group (BMI < 18.5) (p<0.001). Multivariable-adjusted mean levels of plasma BNP were shown in Fig. 1 for each BMI category. For both sexes, adjusted BNP levels decreased in a stepwise fashion across categories of increasing BMI (p<0.001 for trend for all comparisons).

For logistic regression analysis, the BNP levels were considered as a categorical variable, pooling the subjects into two distinct groups: <18.4 pg/mL (nor-

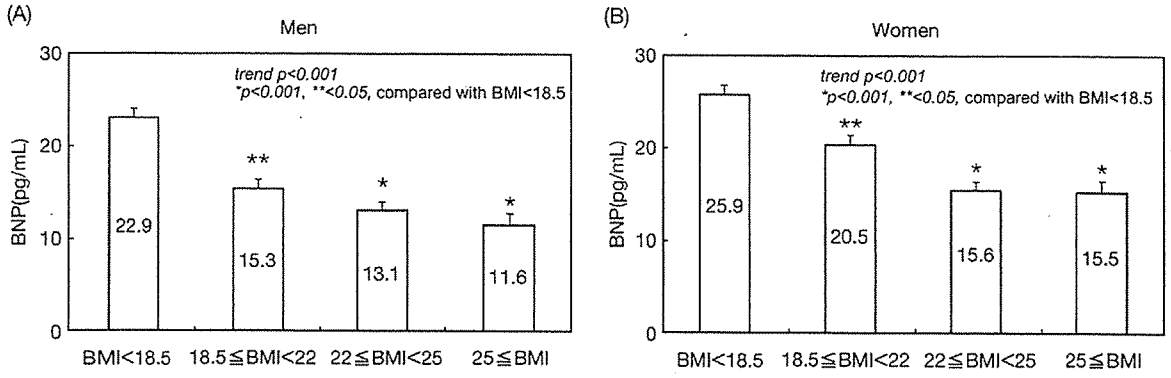

Fig. 1 Adjusted BNP levels stratified by BNP categories. Mean levels and SE of BNP for men (A) and women (B) are shown. Covariates used for adjustment are listed in Table 2.

mal) and ≧ 18.4 pg/mL (abnormal); the same covariates were evaluated in these models as in the linear regression models described above. The adjusted odds ratios of having normal BNP levels are shown in Table 3. After multivariable adjustment, highest BMI (25 kg/m^2 <BMI) was associated with having a 2.1- to 2.3-fold increase in the odds of having normal BNP levels (p<0.001). Overall, for each 1 unit increase in BMI, there was a 11% to 16% greater chance of having normal BNP (p<0.01).

Association between Body Composition and BNP Levels

Results of multivariable regression models relating plasma BNP levels with various obesity related factors are shown in Table 4. Model 1 used BMI as a measure of obesity, and model 2 replaced BMI with percent of body fat. In model 3, percent body fat was replaced by body fat mass and lean body mass. Model 4 replaced BMI with skin fold thickness, and Model 5 replaced BMI with waist circumferences. After adjustment for the same covariates as in Table 2, inverse associations were confirmed between percent of body fat, body fat mass, skin fold thickness and waist circumferences and BNP levels in both sexes (all p<0.01). However, the inverse association with lean body mass was not significant for BNP (p=0.188 in men and p=0.079 in women).

Discussion

In the present study, we showed that higher BMI was significantly associated with lower plasma BNP levels in a general Japanese population. The finding is not attributable to underlying differences in cardiovascular risk factors between obese and non-obese subjects. We also showed that the inverse association between body fat mass, skin fold thickness and waist circumferences and BNP. This is the first report that analyzes the relations between BNP levels and various obesity related factors.

Several studies, including large, population-based cohorts [7, 8], have demonstrated that BMI was inversely correlated with BNP levels in patients with heart failure [9-12]. In the Dallas Heart Study [8], they focused on the body composition instead of BMI and showed an inverse association between plasma BNP and lean mass. However, these studies were conducted mostly in Western countries, where BMI is much higher than in other parts of the world.

In this study, where the average BMI levels (around 23 kg/m^2) was much lower in comparison with the general Western population (around 28 kg/m^2) [8], the association between higher BMI and lower BNP levels was observed after multivariable adjustment. Furthermore, we divided adiposity into its fat and lean mass components and found that fat mass was responsible for the association between higher BMI and lower BNP levels.

As it was already suggested, the natriuretic peptide system and adiposity are closely linked [18, 19]. Natriuretic peptide clearance receptors (NPR-C) are abundant in adipose tissue [18], and thus, it is suggested that adipocytes participate in a removal of BNP from circulation, which leads to the lower plasma BNP levels in obese patients. Furthermore, the Framingham Heart Study [7] showed that obese in-

Table 2 Multivariable linear models of plasma log BNP.

Models	Men		Women	
	β-coefficient (SE)	*p* value	β-coefficient (SE)	*p* value
Continuous BMI, per 1kg/m^2	-0.022 (0.005)	<0.001	-0.021 (0.004)	<0.001
BMI categories				
BMI < 18.5	Referent	-	Referent	-
18.5≦BMI<22	-0.087 (0.033)	0.009	-0.050 (0.019)	0.008
22≦BMI<25	-0.122 (0.032)	<0.001	-0.109 (0.019)	<0.001
25≦BMI	-0.148 (0.034)	<0.001	-0.112 (0.021)	<0.001

The multiplicative effect on plasma BNP levels can be estimated by exponentiating the β-coefficient. For instance, highest BMI (BMI ≦ 25) is associated with a 29% reduction in BNP levels in men, because $10^{(-0.148)} = 0.71$. All models are adjusted age, systolic blood pressure, pulse rate, serum creatinine, and left ventricular hypertrophy.

Table 3 Influence of BMI on odds of having normal plasma BNP levels (< 18.4 pg/mL).

BMI categories	Men		Women	
	Odds ratio (95% CI)	*p* value	Odds ratio (95% CI)	*p* value
BMI < 18.5	1.00 (referent)	-	1.00 (referent)	-
18.5≦BMI<22	1.66 (1.10-2.56)	0.019	1.33 (1.03-1.74)	0.033
22≦BMI<25	2.08 (1.38-3.19)	0.001	1.99 (1.52-2.63)	<0.001
25≦BMI	2.25 (1.47-3.51)	<0.001	2.13 (1.59-2.88)	<0.001
BMI (continuous), per 1kg/m^2	1.11 (1.04-1.18)	0.001	1.16 (1.11-1.23)	<0.001

Multivariable logistic regression models for low BNP levels are adjusted for age, systolic blood pressure, pulse rate, serum creatinine, and left ventricular hypertrophy.

Table 4 Multivariable associations between obesity related factors and BNP.

Models	Men		Women	
	β-coefficient (SE)	*p* value	β-coefficient (SE)	*p* value
Model 1				
BMI	-0.002 (0.005)	<0.001	-0.021 (0.004)	<0.001
Model 2				
Percent of body fat	-0.012 (0.003)	<0.001	-0.011 (0.002)	<0.001
Model 3				
Body fat mass	-0.014 (0.003)	<0.001	-0.013 (0.003)	<0.001
Lean body mass	0.004 (0.003)	0.188	0.007 (0.004)	0.079
Model 4				
Skinfold thickness	-0.007 (0.002)	<0.001	-0.004 (0.001)	0.001
Model 5				
Waist circumference	-0.006 (0.002)	<0.001	-0.006 (0.001)	<0.001

The multiplicative effect on plasma BNP levels can be estimated by exponentiating the β-coefficient. All models are adjusted for age, systolic blood pressure, pulse rate, serum creatinine, and left ventricular hypertrophy.

dividuals had higher odds of having low plasma N-terminal proANP. In the Dallas Heart Study [8], the association between higher BMI and lower NT-proBNP was observed. Since both N-terminal proANP and N-terminal proBNP are not cleared by clearance receptors, the findings of reduced N-terminal proANP levels and N-terminal proBNP levels in obese individuals indicate the mechanism other than the adipocyte clearance of the peptides exists. Recent investigations have raised the possibility that the relation between fat and BNP is bidirectional. Adipocytes also express natriuretic peptide receptor-A (NPR-A), which mediate the biologic effects of ANP and BNP [18]. Investigators have demonstrated activation of NPR-A on adipocytes induces lipolysis [19]. Thus, low BNP levels may lead to reduced lipolysis, additionally perpetuating the obese state.

Several limitations of our study deserve comment. First, since our study was cross-sectional study, we cannot demonstrate the cause-effect relation between low plasma BNP and obesity related factors. Second, plasma BNP levels are under the calculable levels of the assay detection limits in 9.0% of all subjects. Misclassification of BNP levels above and below the detection limit would be expected to cause a conservative bias. To overcome the potential bias, we also used logistic regression analyses to account for the left censoring of the BNP distribution. Finally, we cannot exclude the possibility that obese individuals might have had better cardiac function. However, since many previous studies suggest that obesity has been consistently associated with left ventricular hypertrophy [20, 21], dilatation [22] and the increase in the risk of overt heart failure [23], the possibility is highly unlikely.

In conclusion, higher BMI was associated with lower BNP levels in a general Japanese population, even after adjusted for relevant factors. Body fat mass was responsible for this relationship. Further studies will be needed to explore the underlying mechanism.

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High prevalence of early repolarization in short QT syndrome

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BACKGROUND Short QT syndrome (SQTS) is characterized by an abnormally short QT interval and sudden death. Due to the limited number of cases, the characteristics of SQTS are not well understood. It has been reported recently that early repolarization is associated with idiopathic ventricular fibrillation and the QT interval is short in patients with early repolarization.

OBJECTIVE The purpose of this study was to study the association between early repolarization and arrhythmic events in SQTS.

METHODS The study consisted of three cohorts: SQTS cohort (N = 37), control cohort with short QT interval and no arrhythmic events (N = 44), and control cohort with normal QT interval (N = 185). ECG parameters were compared among the study cohorts.

RESULTS Heart rate, PR interval, and QRS duration were similar among the three study cohorts. Early repolarization was more common in the SQTS cohort (65%) than in the short QT control cohort (30%) and the normal QT control cohort (10%). Duration from T-wave peak to T-wave end was longer in the SQTS cohort than in the short QT control cohort, although QT and corrected QT intervals were similar. In the SQTS cohort, there were more males among patients with arrhythmic events than in those with a family history but without arrhythmic events. In multivariate models, early repolarization was associated with arrhythmic events in the SQTS cohort. ECG parameters including QT and QTc intervals were not associated with arrhythmic events in the SQTS cohort.

CONCLUSION There is a high prevalence of early repolarization in patients with SQTS. Early repolarization may be useful in identifying risk of cardiac events in SQTS.

KEYWORDS Arrhythmia; Electrocardiogram; QT interval; Repolarization; Sudden death

ABBREVIATIONS **QTc** = corrected QT interval; **SQTS** = short QT syndrome

(Heart Rhythm 2010;7:647–652)

Introduction

The short QT syndrome (SQTS) is characterized by an abnormally short QT interval and increased risk of ventricular fibrillation and sudden death.[1,2] Similar to other arrhythmia syndromes, such as long QT syndrome and Brugada syndrome,[3] SQTS is a genetically heterogeneous disease, and, to date, five responsible genes encoding different ion channels have been identified.[3–7] Some inherited arrhythmia syndromes may share genetic backgrounds that result in overlapping arrhythmia phenotypes.[3]

Although early repolarization is generally considered benign,[8] it has been reported recently that early repolarization is associated with increased risk for sudden cardiac death in patients with idiopathic ventricular fibrillation.[9–12] Haissaguerre et al[9] reported that, among patients with idiopathic ventricular fibrillation, the QT interval was shorter in patients with early repolarization than in those without, suggesting an association between early repolarization and QT interval shortening. Evidence that mutations in calcium channel genes are associated with Brugada-type ST-segment elevation and abnormally short QT intervals further suggests a relationship between early phase repolarization abnormalities and short QT interval.[4] Here we report on our

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study of the prevalence of early repolarization and its association with arrhythmic events in SQTS.

Methods

This cooperative study consisted of three cohorts. (1) *SQTS cohort* included patients with SQTS referred to our institutions and patients with SQTS from previous reports. The diagnosis of SQTS was made if a patient with a short QT interval [corrected QT interval (QTc) by Bazett formula ≤330 ms] had an arrhythmic event including documented ventricular fibrillation, resuscitated sudden cardiac death, and syncope and/or had a family history of SQTS, or if a patient with a short QT interval (QTc ≤360 ms) had mutations in ion channel genes responsible for SQTS.[3,13] We searched in the electronic databases PubMed, EMBASE, and Cochrane for all published studies that examined patients with SQTS. The search was limited to the end of June 2009. Published studies were considered eligible if they included clinical characteristics of the patients and ECGs. All ECGs from patients reported in the literature were reanalyzed. Electrophysiologic study was performed in patients with SQTS based on the indication of each institution. (2) *Control cohort with short QT interval* (QTc ≤330 ms) *and no arrhythmic events* was selected from among 86,068 consecutive ECGs stored on the ECG database at Niigata University Medical and Dental Hospital from May 7, 2003 to July 2, 2009. Subjects who did not have arrhythmic events or cardiovascular disease and were not taking any medication were included in this cohort. (3) *Control cohort with normal QT interval* was also selected from the ECG database. This cohort consisted of subjects who were matched to the SQTS cohort for gender and age. Subjects who had normal QT interval (360–440 ms) and did not have cardiovascular disease or were not taking any medication were included in this cohort. Subjects with Brugada-type ST-segment elevation were excluded from all study cohorts.[3,9]

QT intervals were measured on lead V_2 with the tangent methods for determination of QT_{end} using a semi-automated digitizing program with electronic calipers by an experienced observer blinded to the clinical details of all subjects included in this study.[14,15] Early repolarization was defined as elevation of the J point noted as either as QRS slurring or notching ≥0.1 mV in more than two leads.[9]

Differences in parameters were analyzed using multivariable logistic regression models when SQTS cohort and control cohort with short QT interval were compared and analyzed using conditional logistic regression models when SQTS cohort and control cohort with normal QT interval were compared. All statistical analyses were performed with SPSS (version 12.0, SPSS, Inc., Chicago, IL, USA). Two-sided $P < .05$ was considered significant. Values are expressed as mean ± SD. The study protocol was approved by the Ethics Committee of Niigata University School of Medicine. To determine interobserver variability, a second observer made independent blinded QT interval determinations of all study subjects with short QT interval.

Results

Thirty-seven patients with SQTS were identified: 12 from our institutions and 25 reported in the literature,[2,5,6,14,16–25] Forty-four control subjects with short QT interval and 185 control subjects with normal QT interval also were identified (Table 1). The SQTS cohort consisted of 25 (68%) patients with symptoms, including 14 with cardiac arrest (3 sudden death, 11 resuscitated) and 11 with syncope. Genetic screening identified mutations in ion channels in 7 (41%) of 17 probands who were genetically screened (2 *KCNQ1*, 4 *KCNH2*, 1 *KCNJ2*). Among patients in our institutions and those reported in the literature, there was no difference with regard to gender, age, prevalence of family history, QT or QTc interval, or inducibility of ventricular tachyarrhythmia by electrical programmed stimulation.

Heart rate, PR interval, and QRS duration in the SQTS cohort were not different among patients in either the short QT control cohort or the normal QT control cohort (Table 1). QT and corrected QT intervals were shorter in the SQTS and short QT control cohorts than in the normal QT control cohort. Early repolarization occurred in 24 (65%) patients with SQTS (Figure 1). Interobserver variability between two investigators was 8.6 ms (95% confidence interval −0.5 to 17.7 ms) for QT interval and 9.0

Table 1 ECG parameters of study cohorts

	Patients with SQTS (N = 37)	Subjects with short QTc (N = 44)	Versus subjects with short QTc*		Subjects with normal QTc† (N = 185)	Versus subjects with normal QTc	
			OR (95% CI)	*P* value		OR (95% CI)	*P* value
Male gender [N (%)]	27 (73)	34 (77)	2.84 (0.72–11.2)	.14	135 (73)	—	—
Age (years)	30 ± 19	47 ± 23	1.05 (1.02–1.08)	.001	30 ± 19	—	—
Heart rate (bpm)	69 ± 393	65 ± 398	1.00 (1.00–1.01)	.3	70 ± 327	1.00 (1.00–1.00)	0.70
PR interval (ms)	138 ± 19	153 ± 38	1.01 (0.99–1.03)	.54	143 ± 24	0.99 (0.97–1.01)	0.18
QRS interval (ms)	86 ± 7	84 ± 8	0.97 (0.91–1.04)	.38	85 ± 7	1.01 (0.96–1.06)	0.74
QT interval (ms)	286 ± 36	286 ± 15	0.99 (0.97–1.01)	.28	367 ± 36	0.97 (0.96–0.98)	<0.001
QTc (ms)	308 ± 29	299 ± 21	0.98 (0.96–1.00)	.06	399 ± 24	0.97 (0.97–0.98)	<0.001

CI = confidence interval; OR = odds ratio; QTc = corrected QT interval; SQTS = short QT syndrome.

*Models were adjusted for gender and age.

†Gender and age were matched between patients with SQTS and subjects with normal QT interval.

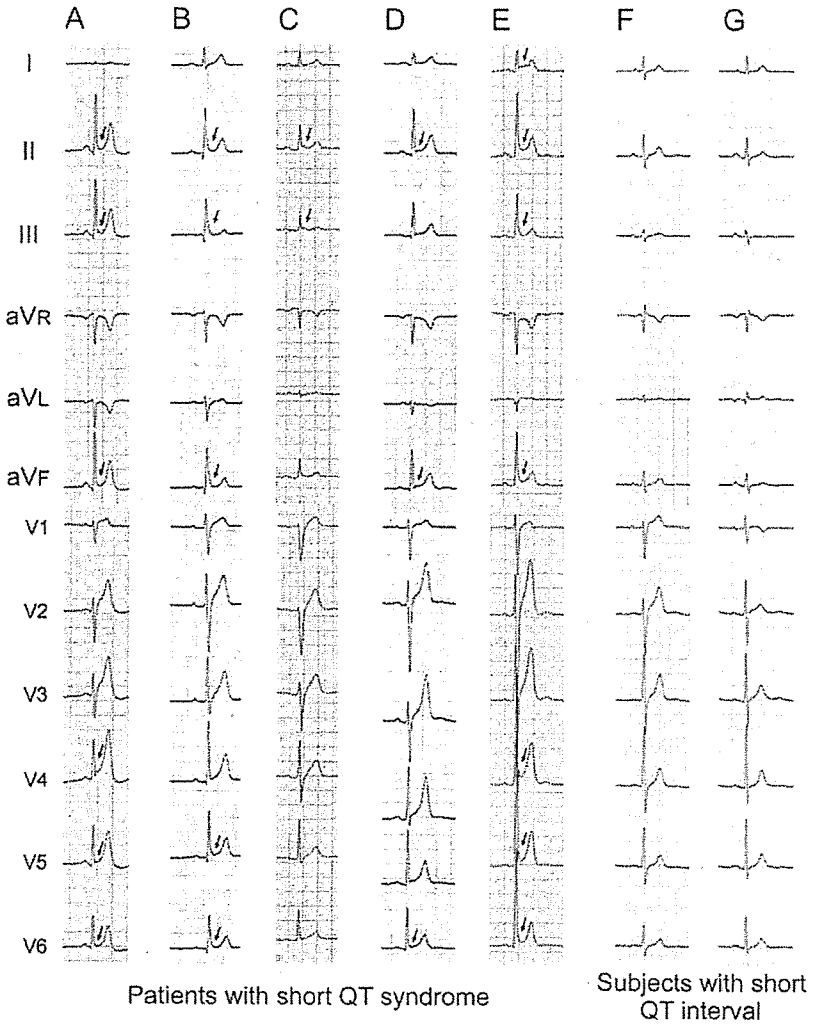

Figure 1 Early repolarization in short QT syndrome. ECGs were recorded from patients with short QT syndrome (**A:** 61-year-old woman; **B:** 30-year-old man; **C:** 38-year-old man; **D:** 31-year-old man; **E:** 22-year-old man) and control subjects with a short QT interval (**F:** 23-year-old man; **G:** 44-year-old woman). In each patient with short QT syndrome, early repolarization was evident in the inferolateral leads (*arrows*).

ms (95% confidence interval −0.6 to 18.7 ms) for QTc interval. The frequency of early repolarization was not different between patients in our institutions and those reported in the literature. Early repolarization was present in the inferior leads (II, III, aVF) in 9 patients, in the lateral leads (I, aVL, V_4–V_6) in 6 patients, and in both the inferior and lateral leads in 9 patients. Of 10 probands with early repolarization genetically screened, mutations were identified in 3 patients (1 *KCNQ1*, 2 *KCNH2*). Early repolarization was more common in the SQTS cohort than in the short QT control and normal QT control cohorts (Figure 2).

The association of early repolarization with arrhythmic events then was studied in patients with SQTS. In the SQTS cohort, there were more males among patients with arrhythmic events than among those with a family history but without arrhythmic events (Table 2). In multivariate models adjusted for gender and age, early repolarization was associated with arrhythmic events, although ECG parameters including QT and QTc intervals were not associated with arrhythmic events. Early repolarization remained associated with arrhythmic events after adjustment for age, gender, and QTc interval (P = .001). Electrophysiologic study performed in 18 patients with SQTS revealed no difference in inducibility of ventricular tachyarrhythmia between patients with arrhythmic events (73%) and those without arrhythmic events (71%).

QT interval parameters were compared between SQTS and short QT control cohorts because some of the parameters recently have been associated with SQTS.[26] Interval from T-wave peak to T-wave end (T_{peak} to T_{end}) was longer in the SQTS cohort than in the short QT control cohort even after heart rate correction using the Bazett formula, whereas QT interval, QTc interval, and interval from Q-wave to T-wave peak (QT_{peak}) were not different between the two cohorts (Table 3). Ratio of T_{peak} to T_{end} per QT was larger in the SQTS cohort than in the short QT control cohort.

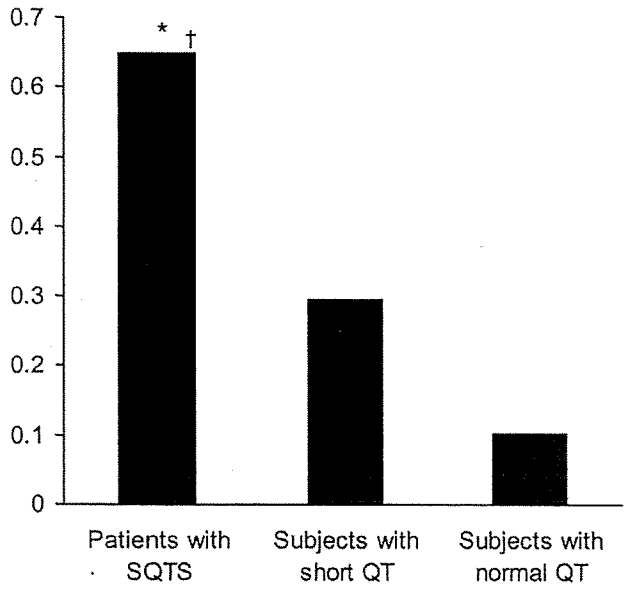

Figure 2 Frequency of early repolarization. Odds ratios (95% confidence intervals) for early repolarization in patients with short QT syndrome (SQTS) were 5.64 (1.97–16.15) and 16.58 (7.2–38.21) versus subjects with short QT interval and those with normal QT interval, respectively. *P = .001 vs subjects with short QT interval. †P <.001 vs subjects with normal QT interval.

Discussion

SQTS is a recently discovered, very rare disease with an increased risk of sudden death.[2] Due to the limited number of cases, the characteristics of SQTS are not well understood. Therefore, we conducted a cooperative analysis of ECGs from patients with SQTS in our institutions and those reported in the literature and found that early repolarization is common in SQTS.

Early repolarization is a common ECG finding. It is present in 1% to 13% of the general population and usually is considered as a normal variant due to its benign long-term prognosis.[8,11,27–29] However, increasing evidence suggests that early repolarization is associated with arrhythmia.[9,27,30–34] Since 1985, we and other investigators have reported an association between early repolarization (or late depolarization) and sudden cardiac death.[30–32] A multicenter study including our institution recently showed that early repolarization is present in one third of patients with idiopathic ventricular fibrillation.[9] Early repolarization is associated with increased risk of sudden cardiac arrest in idiopathic ventricular fibrillation, and the amplitude of early repolarization increases before development of arrhythmic events.[9,10] In Brugada syndrome, which is characterized by J-wave and ST-segment elevation in the right precordial leads on ECG and sudden cardiac death,[3] early repolarization in the inferolateral leads is not uncommon and is associated with arrhythmic events,[34] although another report has shown negative results.[33] In our study, early repolarization in the inferolateral leads was frequently found in SQTS and, more importantly, was associated with arrhythmic events in SQTS. In addition to arrhythmia syndromes unassociated with structural heart disease, a high frequency of early repolarization in arrhythmogenic right ventricular dysplasia/cardiomyopathy has been reported.[27]

It has been suggested that SQTS and idiopathic ventricular fibrillation share clinical characteristics.[23] Short QT interval is frequently found in idiopathic ventricular fibrillation,[23] and QT interval is relatively short in patients with idiopathic ventricular fibrillation who have early repolarization.[9] Spontaneous and inducible ventricular fibrillation can be initiated by short-coupled premature ventricular beat in SQTS and idiopathic ventricular fibrillation.[21,35,36] The efficacy of isoproterenol and quinidine has been reported for both arrhythmia syndromes,[21,37] although the arrhythmogenic effects of isoproterenol in an experimental model of SQTS have been reported.[38] Our study showing an association of early repolarization with SQTS further supports the presence of common arrhythmogenic substrates in SQTS and idiopathic ventricular fibrillation.

A precise mechanism for ventricular fibrillation in SQTS is not known, but characteristic ECG abnormalities may reflect arrhythmogenicity. A prior study showed that the interval from T-wave peak to T-wave end is relatively long in SQTS, and our study replicated the results.[26] T-wave peak to T-wave end interval is considered to reflect transmural dispersion of repolarization, and relative prolongation of the interval in SQTS may indicate a high vulnerability to ventricular fibrillation.[39] An experimental model of SQTS

Table 2 Characteristics of SQTS patients with and those without arrhythmic events

	Patients with arrhythmic events (N = 25)	Patients without arrhythmic events (N = 12)	OR (95% CI)	*P* value
Male gender [N (%)]	21 (84)	6 (50)	10.44 (0.85–127.48)	.07
Age (years)	30 ± 19	23 ± 18	1.05 (0.99–1.12)	.13
Heart rate (bpm)	69 ± 393	76 ± 473	1.00 (1.00–1.01)	.38
PR interval (ms)	138 ± 19	134 ± 18	0.99 (0.95–1.04)	.84
QRS interval (ms)	86 ± 7	85 ± 10	0.93 (0.82–1.07)	.31
QT interval (ms)	286 ± 36	271 ± 40	1.00 (0.97–1.03)	.75
QTc (ms)	308 ± 29	306 ± 33	0.98 (0.94–1.02)	.33
Early repolarization [N (%)]	22 (88)	2 (17)	46.53 (4.52–478.79)	.001

CI = confidence interval; OR = odds ratio; QTc = corrected QT interval; SQTS = short QT syndrome.
Models were adjusted for gender and age.

Table 3 ECG parameters for study cohorts with short QT interval

	Patients with SQTS	Subjects with short QTc	OR (95% CI)	*P* value
QT_{peak} (ms)	211 ± 37	222 ± 19	0.99 (0.98–1.01)	.37
Corrected QT_{peak}	226 ± 32	234 ± 24	0.99 (0.98–1.01)	.56
T_{peak} to T_{end} (ms)	81 ± 21	67 ± 13	1.08 (1.03–1.13)	<.001
Corrected T_{peak} to T_{end}	89 ± 28	72 ± 17	1.05 (1.02–1.09)	.002
QT_{peak}/QT ratio (%)	27 ± 6	22 ± 4	0.83 (0.73–0.94)	.004

Models were adjusted for gender and age.

CI = confidence interval; OR = odds ratio; QTc = corrected QT interval; SQTS = short QT syndrome.

provides evidence that increased transmural dispersion of repolarization under short QT interval conditions results in ventricular tachyarrhythmia.[38] A tall peaked T wave is one of the characteristic ECG abnormalities in SQTS,[1] but the amplitude of the T wave is not different between patients with SQTS and subjects with short QT interval and no arrhythmic events, suggesting that a tall T wave is associated with a short QT interval but is not associated with arrhythmogenicity.[26] In SQTS, characteristic ECG abnormities are also found in the early repolarization phase. In patients with SQTS, the ECG shows a very short J-point to T-wave peak interval and no flat ST segment.[26] In our study, early repolarization was frequently found in SQTS and was associated with arrhythmic events. Whether the inferolateral J-point elevation reflects late depolarization or early repolarization is controversial, but this pattern has been considered repolarization because of slower inscription, spontaneous changes occurring concurrently with ST segment but not with QRS complexes, and absence of late potentials on signal-averaged ECG.[9,40] Taken together, the finding suggest that abnormalities in the early phase of repolarization create the arrhythmogenic substrate in SQTS.

Sex hormone and gender difference have an important role in the arrhythmia syndromes.[41–43] It is well known that the QT interval is affected by sex hormones, and the QT interval is longer in women than men.[44] Female gender is a risk factor for development of ventricular tachyarrhythmias in both congenital and acquired long QT syndrome.[41,42] On the other hand, Brugada syndrome is more prevalent in men than in women, and the male hormone testosterone is reported to contribute to male predominance in Brugada syndrome.[43] In this study, male gender was associated with arrhythmic events in SQTS and short QT interval was frequently found in men, suggesting a role of sex hormones in SQTS opposite to that in long QT syndrome. Recent evidence that the QT interval can be shortened by anabolic androgenic steroids and testosterone further supports this hypothesis.[45,46]

SQTS is a genetically heterogeneous disease with five responsible genes encoding ion channels: *KCNQ1*, *KCNH2*, *KCNJ2*, *CACNA2D1*, and *CACNB2b*.[3,4] An increase in outward current by gain-of-function mutations in potassium channels or a decrease in inward current by loss of function mutations in calcium channels may be responsible for SQTS.[3,4] Early repolarization was found in patients with mutations in *KCNQ1* and *KCNH2* and in those without mutations in the known genes, suggesting a heterogeneous genetic background for the association between short QT interval and early repolarization. To date, mutations in calcium channel genes (*CACNA2D1* and *CACNB2b*) have been identified in three probands with Brugada syndrome associated with a short QT interval, but early repolarization is not present in the inferolateral leads in any of them.[4] A recent study has identified a mutation in *KCNJ8*, an initial responsible gene for idiopathic ventricular fibrillation associated with early repolarization.[47] Although there are some similarities in phenotype between SQTS and idiopathic ventricular fibrillation with early repolarization, a common genetic background has not been identified.

Conclusion

Our study showed a high prevalence of early repolarization in patients with SQTS and an association of early repolarization with arrhythmic events. Early repolarization may be a useful marker for risk stratification of cardiac arrest in SQTS, although further investigation with longitudinal follow-up is required to evaluate our results.

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Triglycerides and non-high-density lipoprotein cholesterol and the incidence of cardiovascular disease in an urban Japanese cohort: The Suita study

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ABSTRACT

Objective: The impact of elevated triglycerides (TG) and non-high density lipoprotein cholesterol (non-HDLC) on the incidence of stroke and myocardial infarction (MI) has not been well evaluated in Asian populations such as in Japan, which have a lower incidence of myocardial infarction, but a higher risk of stroke than Western populations.
Methods: The authors conducted an 11.7-year prospective study ending in 2005 of 5098 Japanese aged 30–79 living in an urban population, initially free of stroke or MI. The relationship between serum lipids and the risk for stroke and MI was determined by dividing the participants into four groups stratified by the combination of serum levels of TG and non-HDLC. The cut-off value was 1.7 mmol/L for TG and 4.9 mmol/L for non-HDLC.
Results and conclusion: The total person-years were 59,774 (27,461 for men and 32,313 for women). During the follow-up period, there were 113 cases of MI and 180 of stoke (with 116 cerebral infarctions). Compared with the low TG/low non-HDLC group, the hazard ratio (95% confidence interval) for MI in the high TG/high non-HDLC group was 2.55 (1.53–4.24) after adjustment for other cardiovascular risk factors. The hazard ratio for cerebral infarction in the high TG alone group was 1.63 (1.03–2.56); however, the risk of cerebral infarction was not significantly increased in the other groups. High serum levels of TG and non-HDLC are both important targets for the prevention of cardiovascular disease in Japan.

1. Introduction

Previous studies suggested that high levels of serum total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) are causal risk factors for coronary artery disease (CAD) [1–4] and possibly for ischemic stroke [5]. However, less attention has been paid to high serum levels of triglycerides (TG) [6–8]. Furthermore, although the US National Cholesterol Education Program Adult Treatment Panel guideline III (NCEP-ATP III) has set goals for non-high-density lipoprotein cholesterol (non-HDLC) after the achievement of LDL-C goals in patients with elevated TG [9], the impact of TG and non-HDLC on the incidence of cardiovascular disease (CVD) has not been evaluated in the Japanese population, which has a lower incidence of CAD but a higher risk of stroke than Western populations [10].

Therefore, our a priori hypothesis was that the coexistence of high serum TG and non-HDLC increases the risk of CAD and stroke in the Japanese population. To investigate this hypothesis, we performed a long-term prospective study in an urban, community-dwelling Japanese population.

2. Methods

2.1. Populations

The Suita study, a cohort study for CVD of urban residents was established in 1989. The details of this study have been described elsewhere [4,11–14]. Briefly, 6485 men and women aged 30–79 years had a baseline survey at the National Cardiovascular Center between September 1989 and March 1994. Of these, a total of 1387 were excluded for the following reasons: past history of coronary heart disease or stroke ($n=210$), lack of participation in the baseline survey ($n=79$), non-fasting visit ($n=166$), use of lipid-lowering agents ($n=125$), missing data ($n=109$), and lost to follow-up ($n=698$). Data from the remaining 5098 participants (2404 men and 2694 women) were included in the analysis. This

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cohort study was approved by the Institutional Review Board of the National Cardiovascular Center.

2.2. Baseline examination

Blood samples were collected after the participants had fasted for at least 10 h. The samples were centrifuged immediately and a routine blood examination was performed that included serum total cholesterol (TC), HDL cholesterol, TG and glucose levels.

Blood pressure was measured in triplicate on the right arm after 5 min of rest by well-trained physicians using a standard mercury sphygmomanometer. The average of the second and third measurements was used for analysis. Hypertension was defined as either a systolic blood pressure (SBP) ≥ 140 mmHg, a diastolic blood pressure (DBP) ≥ 90 mmHg or the use of antihypertensive agents. Diabetes was defined as a fasting serum glucose ≥ 7.0 mmol/L (126 mg/dL), the use of anti-diabetic agents, or both. Height with bare feet and weight in light clothing were measured. Waist circumference (WC) was measured at umbilical level in a standing position. Metabolic syndrome (MetS) was defined using modified NCEP-ATP III criteria [13], of which abdominal obesity was defined according to the International Obesity Task Force central obesity criteria for Asia [15].

Public health nurses obtained information on the smoking, drinking and medical histories.

2.3. Endpoint determination

The endpoint determination was previously reported [4,11–14]. The endpoints of the present study were: (1) the first myocardial infarction (MI) or stroke event; (2) death; (3) leaving Suita city; or (4) December 31, 2005.

The first step in the survey for MI and stroke involved checking the health status of all participants by repeated clinical visits every two years and yearly questionnaires by mail or telephone. In the second step, in-hospital medical records of participants who were suspected of having an MI or stroke were reviewed by registered hospital physicians or research physicians, who were blinded to the baseline information. The criteria for stroke were defined according to the US National Survey of Stroke criteria [16]. For each stroke subtype [i.e., cerebral infarction, intracerebral hemorrhage, and subarachnoid hemorrhage], a definite diagnosis was established based on the computed tomography, magnetic resonance imaging, or autopsy. The criteria for definite and probable MI were defined according to the criteria of the MONiCA (Monitoring Trends and Determinants of Cardiovascular Disease) project [17]. Sudden deaths of unknown origin that occurred within 24 h of the onset were classified as MI in the present study.

2.4. Statistical analysis

The relationship between serum lipids and the risk of MI and stroke was described by dividing the participants into four groups stratified by the combination of serum levels of TG and non-HDLC. We used 1.7 mmol/L (150 mg/dL) of serum TG as a cut-off point for high serum TG according to the classification of NCEP-ATP III [9] and that of the Japan Atherosclerosis Society [3]. The category of non-HDLC ≥4.9 mmol/L (190 mg/dL) was defined as a high serum non-HDLC, which was equivalent to 6.2 mmol/L (240 mg/dL) of TC or 4.1 mmol/L (160 mg/dL) of LDL-C, because non-HDLC was usually 0.8 mmol/L (30 mg/dL) higher than LDL-C [9,18–19].

Continuous variables between groups were compared by analysis of variance and categorical variables were compared by a chi-square test. The hazard ratio (HR) for MI or stroke was calculated using a proportional hazards model adjusted for age, hypertension (dichotomous variable), diabetes, HDL-C, body mass index (BMI), smoking (never-smoked; ex-smoker; current smoker) and drinking (never-drank; ex-drinker; regular drinker) (model 1). Sex-combined analysis with further adjustment for sex was also performed. Another statistical model after replacement of BMI and hypertension with WC and SBP level (continuous variable) was also performed (model 2).

All confidence intervals were estimated at the 95% level and significance was set at a P value of <0.05. The Statistical Package for the Social Sciences (SPSS Japan Inc. version 15.0J, Tokyo, Japan) was used for all the analyses.

3. Results

The median and interquartile range of serum TG in the baseline survey was 1.29 mmol/L (0.90, 1.90) in men and 0.98 mmol/L (0.73, 1.41) in women. The mean baseline serum non-HDLC was 3.93 ± 0.91 mmol/L in men and 4.03 ± 1.03 mmol/L in women.

The means or prevalence of major cardiovascular risk factors in each group stratified by the combination of serum levels of TG and non-HDLC are summarized in Table 1. There was no significant difference in mean age and the prevalence of smoking among the TG and non-HDLC groups for men. There were significant differences in all other variables. Mean BMI, waist circumference and the prevalence of hypertension and diabetes were highest in the high-TG/high non-HDLC group, whereas the values of these parameters were lowest in the low-TG/low-non-HDLC group for both sexes. The prevalence of Mets was much higher in the high-TG groups than in the low-TG groups irrespective of non-HDLC level.

The total person-years were 59,774 (27,461 for men and 32,313 for women), with a mean follow-up period of 11.7 years. During the follow-up period, there were 113 first MIs and 180 first strokes. The strokes consisted of 28 intracerebral hemorrhages, 116 cerebral infarctions, 21 subarachnoid hemorrhages and 15 unclassified cases.

Table 2 shows the number of cases, age and multivariable-adjusted HRs for MI stratified by TG and non-HDLC. Compared with the low TG/low non-HDLC group, the HR for MI in the high TG/high non-HDLC group was 2.05 (95% confidence interval, CI, 1.08–3.90) in men, 3.79 (95% CI, 1.58–9.14) in women and 2.55 (95% CI, 1.53–4.24) in both sexes combined in multivariable adjusted model 1. We did not observe a significant increase in the HR for MI in the other groups. Similar results were observed after replacement of BMI and hypertension with WC and SBP level (model 2).

Table 3 shows the multivariable-adjusted HRs for cerebral infarction stratified by levels of TG and non-HDLC. Compared with the low TG/low non-HDLC group, the HR for cerebral infarction in the high TG alone group (high TG/low non-HDLC group) was 1.45 (95% CI, 0.84–2.50) in men, 2.09 (95% CI, 0.92–4.73) in women and 1.63 (95% CI, 1.03–2.56) in both sexes combined in statistical model 1. There was no significant increase of cerebral infarction in the other groups. Similar results were also observed in statistical model 2.

The incidence of total stroke, intracerebral hemorrhage and subarachnoid hemorrhage was not related to TG and non-HDLC levels in either sex. When the participants were divided into two groups by age (<60 and ≥60), the results of all the analyses listed above were similar in both age groups (data not shown).

4. Discussion

To our knowledge, this is the first cohort study in Japan to clarify the risk for MI and ischemic stroke of high serum level of TG, non-HDLC and both. The risk for MI of both high serum TG and non-HDLC was considerably higher than the risk without both or with only one. This relationship was similarly observed in both men and

Table 1
Means and prevalence of major cardiovascular risk factors in each group stratified by the combination of serum levels of triglycerides (TG) and non-high-density lipoprotein cholesterol (non-HDLC).

Variables	Low TG/low Non-HDLC		Low TG/high Non-HDLC		High TG/low Non-HDLC		High TG/high Non-HDLC		*P* value
Men									
No. of subjects	1532		117		550		205		
Non-HDLC (stratum mean), mmol/L	3.6	(0.7)	5.4	0.4	4.0	0.6	5.5	0.5	
Triglycerides (stratum median), mmol/L	1.0	(0.8, 1.3)*	1.3	(1.0, 1.5)*	2.2	(1.9, 2.9)*	2.4	(2.0, 3.7)*	
Age, years	55.8	(13.5)	57.4	(12.9)	54.8	(12.7)	54.8	(11.8)	0.16
HDLC, mmol/L	1.4	(0.3)	1.3	(0.3)	1.1	(0.3)	1.1	(0.2)	<0.01
BMI, kg/m^2	22.2	(2.8)	23.1	(3.1)	23.8	(2.6)	24.2	(2.6)	<0.01
Waist circumference, cm	80.8	(7.9)	82.7	(8.6)	85.7	(7.0)	86.3	(6.9)	<0.01
Systolic blood pressure, mmHg	127	(21)	129	(20)	130	(20)	132	(21)	<0.01
Diastolic blood pressure, mmHg	78	(12)	79	(12)	81	(11)	82	(11)	<0.01
Hypertension, %	30.0		35.0		36.4		38.0		0.01
Diabetes, %	4.8		4.3		7.5		9.3		0.02
Metabolic syndrome, %**	4.5		4.3		45.1		47.8		<0.01
Smoking, %									
Current smoker	49.9		43.6		53.5		47.3		0.51
Ex-smoker	30.3		35.0		28.4		32.7		
Never-smoker	19.8		21.4		18.2		20.0		
Drinking, %									
Current drinker	76.0		63.2		76.4		69.3		0.02
Ex-drinker	3.6		6.0		2.9		5.4		
Never-drinker	20.4		30.8		20.7		25.4		
Women									
No. of subjects	1956		290		256		192		
Non-HDLC (stratum mean), mmol/L	3.6	(0.7)	5.5	(0.5)	4.2	(0.5)	5.8	(0.8)	
Triglycerides (stratum median), mmol/L	0.9	(0.7, 1.1)*	1.2	(0.9, 1.4)*	2.0	(1.8, 2.4)*	2.4	(2.0, 3.0)*	
Age, years	51.5	(12.9)	59.3	(9.6)	57.9	(11.2)	60.7	(8.8)	<0.01
HDLC, mmol/L	1.5	(0.3)	1.4	(0.3)	1.2	(0.3)	1.1	(0.3)	<0.01
BMI, kg./m2	21.7	(3.1)	22.9	(3.1)	23.6	(3.3)	24.2	(3.1)	<0.01
Waist circumference, cm	75.5	(9.8)	79.8	(9.7)	82.7	(10.0)	83.5	(9.7)	<0.01
Systolic blood pressure, mmHg	121	(21)	131	(21)	132	(21)	137	(21)	<0.01
Diastolic blood pressure, mmHg	73	(12)	79	(12)	79	(12)	80	(13)	<0.01
Hypertension, %	20.4		37.9		37.1		48.4		<0.01
Diabetes, %	2.4		4.5		6.6		7.8		<0.01
Metabolic syndrome, %**	7.5		19.3		66.8		74.5		<0.01
Smoking, %									
Current smoker	11.8		8.6		14.5		16.1		0.04
EX-smoker	3.5		2.8		2.7		6.3		
Never-smoker	84.7		88.6		82.8		77.6		
Drinking, %									
Current drinker	34.9		29.3		28.5		24.5		<0.01
Ex-drinker	1.8		0.3		0.8		4.2		
Never-drinker	63.3		70.3		70.7		71.4		

TG, triglycerides; non-HDLC, non-high-density lipoprotein cholesterol; BMI, body mass index. Brackets indicate standard deviation.
Analysis of variance was used for comparisons of multiple group means and the chi-square test was used to compare proportions.

* Inter-quartile range.

** MetS was defined using modified NCEP-ATP III. Abdominal obesity was defined as a waist circumference ≥0.90 m in men and ≥ 0.80 m in women. High blood pressure was defined as average systolic/diastolic blood pressures of ≥ 130/85 mm Hg and/or current medication for hypertension. High triglyceride was defined as serum triglycerides of ≥1.7 mmol/L. Low HDL cholesterol was defined as serum HDL cholesterol levels of <1.03 mmol/L in men and of <1.29 mmol/L in women. High blood glucose was defined as fasting blood glucose of ≥6.1 mmol/L and/or current use of anti-diabetic medication. MetS was defined as the presence of three or more of these components.

women. In contrast, the risk for ischemic stroke was highest in the participants with high TG alone.

TG-rich lipoproteins have been shown to be atherogenic, and thus, they are associated with coronary atherosclerosis [9,19–20]. As NCEP-ATP III pointed out [9], elevated non-HDLC is a good therapeutic target in patients with high TG, because the serum concentration of non-HDLC reflects not only LDL-C but also the cholesterol content of all other TG-rich and apolipoprotein B containing lipoproteins, such as very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), small dense LDL particles and their remnant lipoproteins [19–20]. In the Helsinki Heart study [21], most of the risk for coronary heart disease (CHD) was confined to participants with high levels of both TG and LDL-C. In the West of Scotland Coronary Prevention Study [22], a higher incidence of CHD was observed in men in both the pravastatin and placebo groups when TG was at or above the median level. Pischon et al. suggested that TG added significant information to non-HDLC for CAD risk prediction in a nested case-control study [23]. Our findings are consistent with previous studies.

Similar to previous studies in Japan [4,10], we found no association between non-HDLC and cerebral infarction even in the presence of high serum TG, which may be due to a lower prevalence of atherothrombotic infarction than in Western populations. The ARIC study indicated that TC was associated with increased risk of non-lacunar, non-embolic stroke (atherothrombotic infarction), but not with lacunar or embolic stroke [24]. A recent report from a Japanese rural population showed that LDL-C is a risk factor for only atherothrombotic infraction [25]. Unfortunately, due to the relatively small stroke cases in our study, we were not able to demonstrate an association between any subtype of cerebral infarction and non-HDLC.

It is not clear why participants with high TG alone showed the increased risk for cerebral infarction in the present study. In a meta-analysis of 26 cohort studies in Asia-Pacific area, partici-

Table 2
Age and multivariable-adjusted hazard ratios (95% confidence intervals) for myocardial infarction stratified by TG and non-HDLC groups in an 11.7-year prospective study of 5098 Japanese men and women.

	Low TG/low Non-HDLC	Low TG/high Non-HDLC	High TG/low Non-HDLC	High TG/high Non-HDLC
Men				
Person-years	17410	1288	6358	2404
Case, *n*	45	6	11	14
Age adjusted	1.00	1.63 (0.70-3.83)	0.76 (0.39-1.48)	2.74(1.50-5.02)
Model 1[a]	1.00	1.48 (0.62-3.49)	0.63 (0.32-1.26)	2.05(1.08-3.90)
Model 2[b]	1.00	1.55 (0.66-3.66)	0.64 (0.32-1.29)	2.10 (1.10-3.98)
Women				
Person-years	23652	3455	2936	2270
Case, *n*	14	5	6	12
Age adjusted	1.00	1.59 (0.57-4.40)	2.28 (0.88-5.94)	4.88 (2.25-10.6)
Model 1[a]	1.00	1.63 (0.58-4.26)	1.99 (0.71-5.57)	3.79 (1.58-9.14)
Model 2[b]	1.00	1.55 (0.55-4.38)	1.92 (0.69-5.34)	3.18 (1.34-7.52)
Men and women				
Person-years	41062	4743	9294	4674
Case, *n*	59	11	17	26
Age adjusted	1.00	1.51 (0.79-2.89)	1.04 (0.60-1.78)	3.42 (2.15-5.44)
Model 1[a]	1.00	1.42 (0.74-2.74)	0.86 (0.49-1.53)	2.55 (1.53-4.24)
Model 2[b]	1.00	1.45 (0.75-2.79)	0.87 (0.49-1.54)	2.48 (1.49-4.10)

TG, triglycerides; non-HDLC, non high-density lipoprotein cholesterol.
[a] Multivariable adjusted for age, body mass index, hypertension, diabetes, HDL (high-density lipoprotein) cholesterol, cigarette smoking and alcohol intake by a Cox proportional hazard model. Sex was also adjusted in the men and women combined model.
[b] Replacement of body mass index and hypertension as covariates in model 1 with waist circumference and systolic blood pressure level.

pants grouped in the highest fifth of serum TG had a 50% increased risk of stroke compared with those in the lowest fifth [26]. Recent reviews have also concluded that hypertriglyceridemia seems to be a causal risk factor for ischemic stroke [7–8]. However, above-mentioned findings were not able to explain the low incidence of cerebral infarction in the high TG/high non-HDLC group in the present study. An elevated risk for MI might mask the relationship between TG and cerebral infarction; because there would be no further follow-up after a first MI. Another large study concerning about the relationship between serum TG and stroke should be needed.

Recently, we have reported that high serum LDLC and non-HDLC are both associated with an increased risk of MI; and the predictive value of non-HDLC for MI is almost similar to that of LDL-C [4]. However, we did not use serum TG as a covariate to avoid over-adjustment, because difference between serum level of LDLC and non-HDLC was automatically determined by serum TG level when serum LDLC value was calculated by the Friedewald formula [27]. Considering all the findings together, non-HDLC and TG may be recommended as beneficial screening markers for primary prevention of CAD in the Japanese community, as they are less expensive and more convenient because non-HDLC can be calculated irrespective of serum TG level.

The present study has some limitations. First, the single TG and non-HDLC measurement at the baseline survey may have underestimated the relationship between these lipids and cardiovascular disease due to regression dilution bias. Furthermore, we did not evaluate longitudinal trend for each risk factor and its medication status after baseline survey. Especially, hypertriglyceridemia is associated with not only present existence of metabolic components, such as hypertension and diabetes, but also new onset

Table 3
Age and multivariable-adjusted hazard ratios (95% confidence intervals) for cerebral infarction stratified by TG and non-HDLC groups in an 11.7-year prospective study of 5098 Japanese men and women.

	Low TG/low Non-HDLC	Low TG/high Non-HDLC	High TG/low Non-HDLC	High TG/high Non-HDLC
Men				
Person-years	17410	1288	6358	2404
Case, *n*	46	2	22	5
Age adjusted	1.00	0.53 (0.13-2.19)	1.51 (0.91-2.52)	0.99 (0.39-2.51)
Model 1[a]	1.00	0.54 (0.13-2.25)	1.45 (0.84-2.50)	0.92 (0.35-2.38)
Model 2[b]	1.00	0.56 (0.14-2.31)	1.48 (0.86-2.56)	0.75 (0.26-2.14)
Women				
Person-years	23652	3455	2936	2270
Case, *n*	20	8	10	3
Age adjusted	1.00	1.77 (0.78-4.02)	2.62 (1.23-5.60)	0.81 (0.24-2.72)
Model 1[a]	1.00	1.52 (0.66-3.50)	2.09 (0.92-4.73)	0.69 (0.20-2.44)
Model 2[b]	1.00	1.54 (0.67-3.54)	2.10 (0.93-4.73)	0.77 (0.22-2.71)
Men and women				
Person-years	41062	4743	9294	4674
Case, *n*	66	10	32	8
Age adjusted	1.00	1.14 (0.58-2.23)	1.82 (1.19-2.79)	0.94 (0.45-1.95)
Model 1[a]	1.00	1.12 (0.57-2.20)	1.63 (1.03-2.56)	0.79 (0.37-1.69)
Model 2[b]	1.00	1.12 (0.57-2.21)	1.62 (1.03-2.55)	0.69 (0.62-1.88)

TG, triglycerides; non-HDLC, non high-density lipoprotein cholesterol.
[a] Multivariable adjusted for age, body mass index, hypertension, diabetes, HDL (high-density lipoprotein) cholesterol, cigarette smoking and alcohol intake by a Cox proportional hazard model. Sex was also adjusted in the men and women combined model.
[b] Replacement of body mass index and hypertension (prevalence) as covariates in model 1 with waist circumference and systolic blood pressure levels.

of them in the future [28,29]. Second, we did not measure serum apolipoprotein B (apoB) [22], apolipoprotein A1 (ApoA1) and LP(a) [30], which some previous studies have shown to be strong risk factors for CAD [22]. Third, a recent study indicated that non-fasting TG is a better predictor of CAD than fasting TG [31]. However, in a large individual based meta-analysis in the Asia-Pacific region [26], most blood samples were collected during fasting, and there was a significant positive relationship between serum TG and CAD or stroke.

In conclusion, a combination of higher serum levels of TG and non-HDLC is associated with an increased risk of MI in a Japanese population. Furthermore, the risk for ischemic stroke was highest in the participants with high TG alone; however, further research should be needed. High serum levels of TG and non-HDLC are both important targets for the prevention of cardiovascular disease, which requires evidence-based guidelines for management in the primary care setting.

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