Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION



**Role of Jagged1 in Arterial Lesions After Vascular Injury**

Xiaojing Wu, Yunzeng Zou, Qi Zhou, Lan Huang, Hui Gong, Aijun Sun, Kaoru Tateno, Ken-ichi Katsube, Freddy Radtke, Junbo Ge, Tohru Minamino and Issei Komuro

*Arterioscler Thromb Vasc Biol* 2011, 31:2000-2006: originally published online June 16, 2011
doi: 10.1161/ATVBAHA.111.225144
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 1079-5642. Online ISSN: 1524-4636

# Role of Jagged1 in Arterial Lesions After Vascular Injury

Xiaojing Wu, Yunzeng Zou, Qi Zhou, Lan Huang, Hui Gong, Aijun Sun, Kaoru Tateno, Ken-ichi Katsube, Freddy Radtke, Junbo Ge, Tohru Minamino, Issei Komuro

***Objective***—Impaired regeneration of endothelial cells (EC) and overactivity of vascular smooth muscle cells (VSMC) are hallmarks of the arterial lesions associated with aging. The occurrence of 2 opposing cellular processes in the same arterial milieu makes pharmaceutical treatment difficult to develop. We previously reported that endothelial expression of a Notch ligand (Jagged1) was reduced in aged animals and that growth of the neointima was enhanced in these animals.

***Methods and Results***—Similar to aged animals, Tie2-cre$^{+}$ Jagged1$^{lox/+}$ mice (with heterologous knockout of Jagged1 in EC) showed exaggerated intimal and medial thickening after carotid artery ligation. Unexpectedly, these mice showed little increase of Jagged1 expression not only in EC but also in VSMC, in contrast to a significant upregulation of Jagged1 in wild-type arteries after ligation. Coculture of VSMC with Jagged1-null EC resulted in the transition of VSMC from the contractile to the synthetic phenotype, along with decreased Jagged1 expression by VSMC. Conversely, overexpression of Jagged1 by EC or VSMC was shown to prevent the unfavorable phenotypic transition of VSMC, under both monoculture and coculture conditions.

***Conclusion***—These findings suggest a unidirectional effect of Jagged1 on both EC and VSMC that contributes to inhibition of arterial lesions after vascular injury. Our data also indicate that Jagged1 may be a novel therapeutic target for aging-related vascular diseases. (***Arterioscler Thromb Vasc Biol.* 2011;31:2000-2006.)**

**Key Words:** aging ■ endothelium ■ smooth muscle cell ■ vascular injury

Ageing is an independent risk factor for the development of cardiovascular disease.[1] Age-associated remodeling of the vascular wall leads to intimal and medial thickening as well as increased vascular stiffness.[2,3] Although both impaired regeneration of endothelial cells (EC) and enhanced proliferation of vascular smooth muscle cells (VSMC) contribute to the progression of age-associated vascular remodeling, how aging regulates these opposing processes in the same arterial milieu remains largely unknown. Moreover, the contrary behavior of these 2 types of cells makes pharmaceutical treatment of aging-associated vascular disease difficult to achieve. For instance, although drug-eluting stents for coronary intervention can lessen restenosis by inhibiting VSMC proliferation, their inhibition is nonselective and can also disturb endothelial repair, forcing patients to prolong the use of dual antiplatelet therapy to prevent late thrombosis.[4] An agent that promotes both EC and VSMC protection could be ideal to overcome such issues.

Among factors that influence the functions of both EC and VSMC and are also related to the ageing process, we focused on Notch. This is an evolutionarily conserved intercellular signaling pathway, which has been shown to contribute to decisions about cellular fate during embryogenesis. Recent investigations have revealed a potential link between Notch signaling and the repair of aged skeletal muscle.[5,6] In the cardiovascular system, Notch signaling is known to be involved in various physiological and pathological processes, including regulation of blood vessel sprouting and branching during angiogenesis and the regulation of arterial and venous differentiation during embryonic vessel formation.[7] There have also been several reports that Notch signaling, in company with its ligand Jagged1, has a role in responses of EC and VSMC during development or postnatal remodeling after vascular injury.[8–10] Thus, we hypothesized that Notch signaling might play a pivotal role in the development and progression of age-related vascular diseases.

We previously reported that endothelial expression of a Notch ligand (Jagged1) was reduced in aged animals, in association with increased VSMC proliferation, and that the formation of neointima was enhanced in these animals.[11] In

Received on: February 8, 2011; final version accepted on: June 7, 2011.

From the Cardiovascular Center of Xinqiao Hospital, Third Military Medical University, Chongqing, China (X.W., L.H.); the Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba, Japan (X.W., K.T., T.M.); Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital and Institute of Biomedical Sciences, Fudan University, Shanghai, China (Y.Z., H.G., A.S., J.G.); the Cardiovascular Department of the Second Affiliated Hospital, Chongqing Medical University, Chongqing, China (Q.Z.); the Department of Molecular Pathology, Graduate School of Tokyo Medical and Dental University, Tokyo, Japan (K.K.); Ecole Polytechnique Fédérale de Lausanne, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland (F.R.); and the Department of Cardiovascular Medicine, Osaka University School of Medicine, Osaka, Japan (I.K.).

Correspondence to Issei Komuro, MD, PhD, Department of Cardiovascular Medicine, Osaka University School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail komuro-tky@umin.ac.jp



*Arterioscler Thromb Vasc Biol* **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.111.225144**

vitro study suggested that EC and VSMC may interact with each other via Notch and that reduced endothelial expression of Jagged1 may be crucial for age-associated vascular remodeling. However, the detailed mechanisms underlying such cell-to-cell interactions remain unclear. More importantly, it is still unknown whether such interactions have any significance in vivo. In the present study, we demonstrate a causative role of endothelial Jagged1 in the occurrence of intimal and medial thickening after vascular injury in vivo. We also provide novel data indicating that endothelial Jagged1 induces VSMC to express Jagged1, presumably through a paracrine mechanism, and that this influences the phenotypic transition of VSMC. These findings suggest a unidirectional influence of Jagged1 on both EC and VSMC that inhibits arterial lesion formation after vascular injury. Our findings support the possibility that Jagged1 may be a novel therapeutic target for aging-related vascular diseases.

## Materials and Methods

### Animals and Carotid Artery Ligation

C57BL/6J mice were purchased from SLC Japan, and the Tie2-cre mice were obtained from Jackson Laboratories. The floxed Jagged-1 mice have been described elsewhere.[12] Deletion of Jagged1 in EC was accomplished by crossing male Tie2-cre$^{+}$ mice with female Jagged1$^{lox/lox}$ mice (Tie2-cre$^{+}$ Jag1 $^{lox/+}$), and the corresponding littermate without Cre transgnene (Tie2-cre$^{-}$ Jag1$^{lox/+}$) served as controls. These mice also served as a donor for the bone marrow transplantation models.[13] The mouse carotid artery was ligated with a 6-0 silk suture just proximal to the carotid bifurcation as previously described.[14,15] All experimental procedures were done according to the guidelines established by Chiba University for animal experiments and all protocols were approved by our institutional review board.

### Morphology Analysis

The carotid arteries were harvested at 4 weeks after the ligation surgery and were then perfusion-fixed with 4% paraformaldehyde and embedded in paraffin. Sections (each 5 $\mu$m thick) at 3 to 4 mm proximal to the ligation site were obtained in each animal. Areas of the lumen, intima, and media were measured in sections stained with hematoxylin and eosin and analyzed with the National Institutes of Health Image Program, as previously described.[14,15]

### Immunofluorescence

The carotid arteries were harvested at 2 weeks after the ligation surgery and were then embedded in OCT compound, frozen, and sectioned. Immunofluorescence for Jagged1 (Santa Cruz Biotechnology: sc-6011), $\alpha$-smooth muscle actin (SMA, Sigma:A2547), and CD31 (BD Pharmingen:558736) was performed by standard procedures.

### Cell Culture and Assay

Primary cultures of human aortic EC (HAEC) and VSMC were from Bio Whittaker (Walkersville, MD). The cells of passage 5 to 7 were used for the experiments. The proliferation assay by MTT[16] and cell count were performed as previously described.[11] Peripheral blood cells of mice were harvested as previously described.[13]

### Retroviral Infection

Retroviral gene transfer of Jagged1 in pLNCX2 vectors (Clontech) was done as previously described.[17] Briefly, retroviral stocks were generated by transient transfection of the HEK-293 packaging cell line and stored at $-80$°C until use. HAEC or VSMC were plated at $4\times10^5$ cells per 100-mm diameter dish 24 hours before infections. For infections, the culture medium was replaced by retroviral stocks supplemented with 8 $\mu$g/mL polybrene (Sigma). At 48 hours after infection, the infected cell populations were selected by culture in 500 $\mu$g/mL G418 for 6 days. After selection, the cells were cultured in fresh complete medium and subjected to experiments. Information about the retroviral vector encoding the Jagged1 gene[18] is available on request.

### Transfection of siRNA

RNA interference was used to knock down the expression of Jagged1 in the EC or VSMC. A small interfering RNA (siRNA) of Jagged1 (sequence: UAAAUGUGAUGUUCGCACAGUUAUC) was designed according to the human Jagged1 sequence (NM_000214), and the oligo was synthesized by Invitrogen. The control siRNA (The BLOCK-iT Alexa FluorR Red Fluorescent Oligo, Cat No. 14750–100) and the transfection reagents (Lipofectamine RNAi Max) were obtained from Invitrogen. After preparation with the siRNA transfection reagent, the siRNA for Jagged1 or the control siRNA was transfected into the cells (at 30% to 50% confluence) according the manufacturer's instructions. To confirm the effect of siRNA on the expression of Jagged1, some of the cells were subjected to Western blot analysis, using an anti-Jagged1 antibody.

### Coculture of HAEC With VSMC

VSMC and HAEC were cocultured, respectively, in cell culture inserts and corresponding companion 6- or 12-well plates (BD Biosciences). HAEC overexpressing Jagged1, or transfected with Jagged1 siRNA, were cultured in complete medium (EGM-2, Lonza) until 90% confluence. VSMC were inoculated onto the insert at a density of $10^5$ cells/mL. Twenty-four hours later, the inserts with VSMC were inserted into the culture dishes of the EC. After 24 hours of incubation with or without 10% serum, VSMC in the upper chamber were harvested for further experiments.

### Western Blotting

Total cell protein was extracted with RIPA buffer (Santa Cruz Biotechnology), and nuclear protein was extracted with NE-PER nuclear extraction reagents (Pierce). The lysates were then separated on SDS-PAGE, transferred to polyvinylidene fluoride membrane, and probed with polyclonal antibody for Jagged1, cleaved Notch1 (Notch intracellular domain, NICD, Cell Signaling, No. 2421,) and actin (Santa Cruz Biotechnology, sc-8432) by standard procedures.

### Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction

RNA was isolated from carotid artery or cultured cells using the RNeasy mini kit (Qiagen). Reverse transcription and real-time polymerase chain reaction (PCR) conditions are described in the online Data Supplement available at http://atvb.ahajournals.org. Relative mRNA expression was calculated with the comparative $C_T$ method and normalized to the expression of endogenous 18s or GAPDH.

### Statistical Analysis

Data are expressed as mean±SEM. Differences were determined either by unpaired Student $t$ test or by 1-way ANOVA followed by a post hoc test to compare the differences between 2 groups. Values of $P<0.05$ were considered significant.

## Results

### Tie2-cre$^{+}$ Jag1$^{lox/+}$ Mice Show Increased Intimal Thickening After Carotid Artery Ligation

Real-time PCR analyses demonstrated that the most prominent Notch receptor in EC was Notch1, and the ligand Jagged1, suggesting a major role for these molecules (Supplemental Figure IA, IB). We previously reported that endothelial expression of Jagged1 decreased with advancing age and the neointimal proliferation was enhanced in aged rats.[11] Likewise, endothelial Jagged1 expression was reduced in

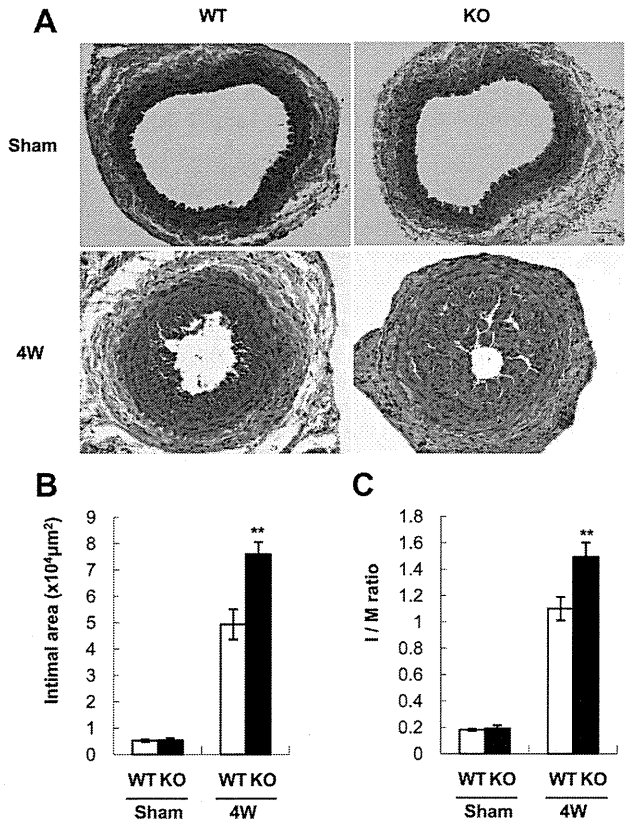



**Figure 1.** Endothelial cell–specific Jagged1 knockout (KO) resulted in intima/media (I/M) thickening after carotid artery ligation. A, Representative photomicrographs of hematoxylin and eosin staining of carotid arteries of Tie2-cre$^{-}$Jag1$^{lox/+}$ mice (wild-type, WT) and Tie2-cre$^{+}$ Jag1$^{lox/+}$ mice (KO), 4 weeks (4W) after ligation (scale bar=50 $\mu$m). B and C, Neointimal area and I/M ratio at 4 weeks after carotid artery ligation. **$P$<0.01 versus WT. Error bars indicate SEM; n=5.

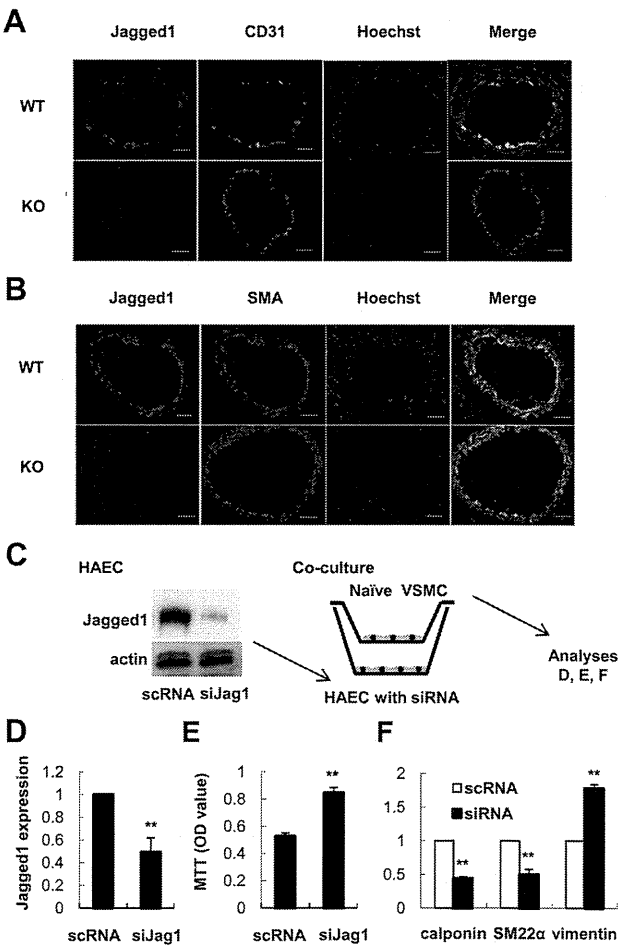



**Figure 2.** Attenuation of endothelial cell (EC) Jagged1 induced vascular smooth muscle cells (VSMC) to decrease Jagged1 expression, in company with synthetic phenotype transition. A and B, Immunofluorescence of frozen sections 2 weeks after the ligation surgery. The sections were stained with Jagged1 and CD31 (A), or Jagged1 and $\alpha$-smooth muscle actin (B). Hoechst 3321 stains nuclei (scale bar, 50 $\mu$m). C through F, Coculture of VSMC with Jagged1 Knockout (KO) EC. C, A schematic model of the coculture and Jagged1 expression of human aortic EC (HAEC) transfected with siRNA for Jagged1 or its control RNA, shown by Western blot. D, Expression of Jagged1 in VSMC after coculture with Jagged1 interfered HAEC, quantified by real-time PCR. E, VSMC proliferation in response to 10% serum as evaluated by the MTT assay after coculture with Jagged1 interfered HAECs for 24 hours. F, Phenotype gene expression in VSMC by real-time PCR after coculture with Jagged1 interfered HAECs for 24 hours. **$P$<0.01 versus scRNA. n=3. WT indicates wild-type.

aged mice (Supplemental Figure IIA, IIB). To investigate whether endothelial expression of Jagged1 had an influence on arterial lesion formation in vivo, we developed endothelial-specific Jagged1 heterozygous deficient (Tie2-cre$^{+}$ Jag1$^{lox/+}$) mice. Expression of Jagged1 mRNA in the intact carotid artery was significantly decreased in Tie2-cre$^{+}$ Jag1$^{lox/+}$ mice compared with that in the carotid artery of control (Tie2-cre$^{-}$ Jag1$^{lox/+}$) mice, falling to 30.29±5.26% of the level in control mice (n=5, $P$<0.01) (Supplemental Figure IIC). In contrast, Jagged1 expression was similar in the carotid arteries of Tie2-cre$^{+}$ Jag1$^{lox/+}$ mice and control mice after the carotid endothelium was removed (n=5, $P$=NS) (Supplemental Figure IID). After carotid artery ligation, the area of the neointima was significantly larger in Tie2-cre$^{+}$ Jag1$^{lox/+}$ mice than in control mice at 28 days after carotid ligation (intimal area: 7.59±0.46×10$^{4}$ $\mu$m$^{2}$ versus 4.93±0.57×10$^{4}$ $\mu$m$^{2}$, n=5, $P$<0.01; N/M 1.49±0.11 versus 1.10±0.09, n=5, $P$<0.01) (Figure 1A through 1C).

## Increase of Jagged1 in VSMC After Carotid Ligation Is Blunted in Tie2-cre$^{+}$ Jag1$^{lox/+}$ Mice

In an attempt to confirm the expression of Jagged1 in mice, we performed double immunofluorescence staining of sections obtained from the animals at 14 days after carotid artery ligation. Using anti-Jagged1 and anti-CD31 antibodies, we found that the expression of Jagged1 in EC was significantly increased in the control mice, whereas (as expected) this increase was minimal in Tie2-cre$^{+}$ Jag1$^{lox/+}$ mice (Figure 2A). Unexpectedly, this was accompanied by marked elevation of Jagged1 expression in the intima and media of the control mice. Double immunofluorescence staining with Jagged1 and SMA revealed that the majority of the Jagged1-expressing cells in the intima and media were VSMC (Figure 2B). More surprisingly, the increase of Jagged1 in VSMC was clearly impaired in the Tie2-cre$^{+}$ Jag1$^{lox/+}$ mice (Figure 2B). Because Jagged1 expression in VSMC should not be genetically affected in Tie2-cre$^{+}$ Jag1$^{lox/+}$ mice, the results

suggested that expression of Jagged1 by VSMC might be regulated through Jagged1 expression by EC, at least in the context of arterial ligation injury.

### Coculture With Jagged1 Knockout EC Results in Jagged1 Attenuation and VSMC Transition

To confirm that endothelial Jagged1 affects the expression of Jagged1 in VSMC and to find out whether this influences the phenotype of VSMC (directing these cells toward neointimal formation or medial hyperplasia), we developed a cell coculture model (Figure 2C). We found that expression of Jagged1 mRNA by VSMC was significantly decreased after coculture with Jagged1 knockdown HAEC (Figure 2D). Although proliferation of VSMC was not affected under the basal conditions without serum stimulation (data not shown), VSMC proliferation determined by the MTT assay was increased after coculture with Jagged1 knockdown HAEC in the presence of 10% serum (scRNA versus siRNA $0.53\pm0.02$ versus $0.84\pm0.04$, n=4, $P<0.01$) (Figure 2E). We then examined the effects of endothelial Jagged1 on expression of phenotypic genes, including calponin or SM22$\alpha$ (contractile VSMC markers) and vimentin (a synthetic VSMC marker). We found that coculture with Jagged1 knockdown EC resulted in decreased expression of both calponin and SMC22$\alpha$ by VSMC, as well as increased expression of vimentin (Figure 2F), indicating phenotypic transition of these cells from the contractile to the synthetic type. Thus, endothelial Jagged1 had a significant influence on Jagged1 expression and phenotypic transition of VSMC.

### Loss of Jagged1 in VSMC Induces Synthetic Transition

To examine the relationship between expression of Jagged1 by VSMC and their phenotypic transition, we studied VSMC monocultures. VSMC were transfected with a small interfering RNA (siRNA) for Jagged1 and with control RNA (scRNA). Western blotting of nuclear protein revealed that the activated form of the Notch1 receptor (NICD; Notch intracellular domain) simultaneously decreased after transfection with Jagged1 siRNA (Figure 3A). Expression of Jagged1 by VSMC did not influence the growth of these cells under basal conditions without stimulation (data not shown), but blocking of Jagged1 expression increased the proliferation of VSMC (as determined by cell counting and the MTT assay) after stimulation with 10% serum for 24 hours (MTT: $0.32\pm0.04$ versus $0.54\pm0.06$, n=6, $P<0.01$; cell counting: $3.57\pm0.33$ versus $5.01\pm0.20\times10^4$ cells, n=4, $P<0.01$) (Figure 3B and 3C). Blocking of Jagged1 expression by VSMC also resulted in decreased expression of calponin and SMC22$\alpha$, whereas there was increased expression of vimentin (Figure 3D). These findings indicate that loss of Jagged1 expression was sufficient to induce the synthetic transition of VSMC.

### Overexpression of Jagged1 Changes VSMC to the Contractile Phenotype

Thus far, our results indicated that loss of Jagged1 expression by EC or VSMC caused these cells to contribute to neointimal formation or medial hyperplasia. Thus, we considered that enhancement of Jagged1 expression might be a novel therapeutic method for such pathological conditions. To further address this issue, we examined whether overexpression of Jagged1 in either VSMC or EC could induce VSMC to cause proliferation and/or synthesis. First, we infected VSMC with a retrovirus encoding Jagged1 (Figure 3E). Overexpression of Jagged1 induced the activation of Notch signaling (Figure 3E) and decreased the proliferation of VSMC (as determined by cell counting and the MTT assay) after stimulation with 10% serum for 24 hours (MTT: $0.45\pm0.04$ versus $0.23\pm0.02$, n=6, $P<0.01$; cell counting: $3.99\pm0.25\times10^5$ versus $2.42\pm0.47\times10^4$ cells, n=4, $P<0.01$) (Figure 3F, G). This also resulted in increased expression of calponin and SMC22$\alpha$ but decreased expression of vimentin (Figure 3H), in contrast to the results of siRNA experiments.

### Coculture With Jagged1-Overexpressing EC Elevates Jagged1 Expression by VSMC and Promotes the Contractile Transition

Finally, we infected HAEC with the Jagged1 retrovirus (Figure 4A). VSMC cocultured with these Jagged1-overexpressing HAEC showed increased expression of Jagged1 mRNA (Figure 4B). Proliferation of VSMC (as determined by the MTT assay) was reduced after coculture with Jagged1-overexpressing HAEC (Figure 4C), whereas expression of calponin and SMC22$\alpha$ was increased significantly, and vimentin expression was markedly attenuated (Figure 4D). Thus, overexpression of Jagged1 in either EC or VSMC inhibited proliferation and synthesis by VSMC, strongly suggesting that enhancement of Jagged1 expression in the vascular wall might prevent neointimal formation or medial hyperplasia after vascular injury (Figure 5).

### Heterozygous Jagged1 Deficiency in Bone Marrow–Derived Cells Has Little Influence on VSMC Phenotype and the Arterial Lesion Formation

The Cre transgene was driven by the Tie-2 promoter, which was predominantly activated in endothelial cells but also in hematopoietic stem cells to some extent.[19] To exclude the possibility that reduced expression of Jagged1 in hematopoietic cells affects neointimal formation in Tie2-cre$^+$ Jag1$^{lox/+}$ mice, we harvested peripheral blood cells from Tie2-cre$^+$ Jag1$^{lox/+}$ mice or control littermates and coincubated them with VSMC in the Boyden chamber. Although we found a modest decrease in platelet counts of the mutant mice, the number and fraction of white blood cells, or the number of red blood cells, did not differ between Tie2-cre$^+$ Jag1$^{lox/+}$ mice and control littermates (Supplemental Figure IIIA). Coculture with blood cells of Tie2-cre$^+$ Jag1$^{lox/+}$ mice did not affect expression of phenotypic genes and Jagged1 by VSMC compared with that with wild-type blood cells (Supplemental Figure IIIB, IIIC). To further confirm these results, we transplanted the bone marrow cells from either wild-type or Tie2-cre$^+$ Jag1$^{lox/+}$ mice into lethally irradiated wild-type mice, followed by carotid artery ligation. We found that heterozygous Jagged1-deficiency in bone marrow–derived cells had little influence on the arterial lesion formation after injury (Supplemental Figure IIID, IIIE).

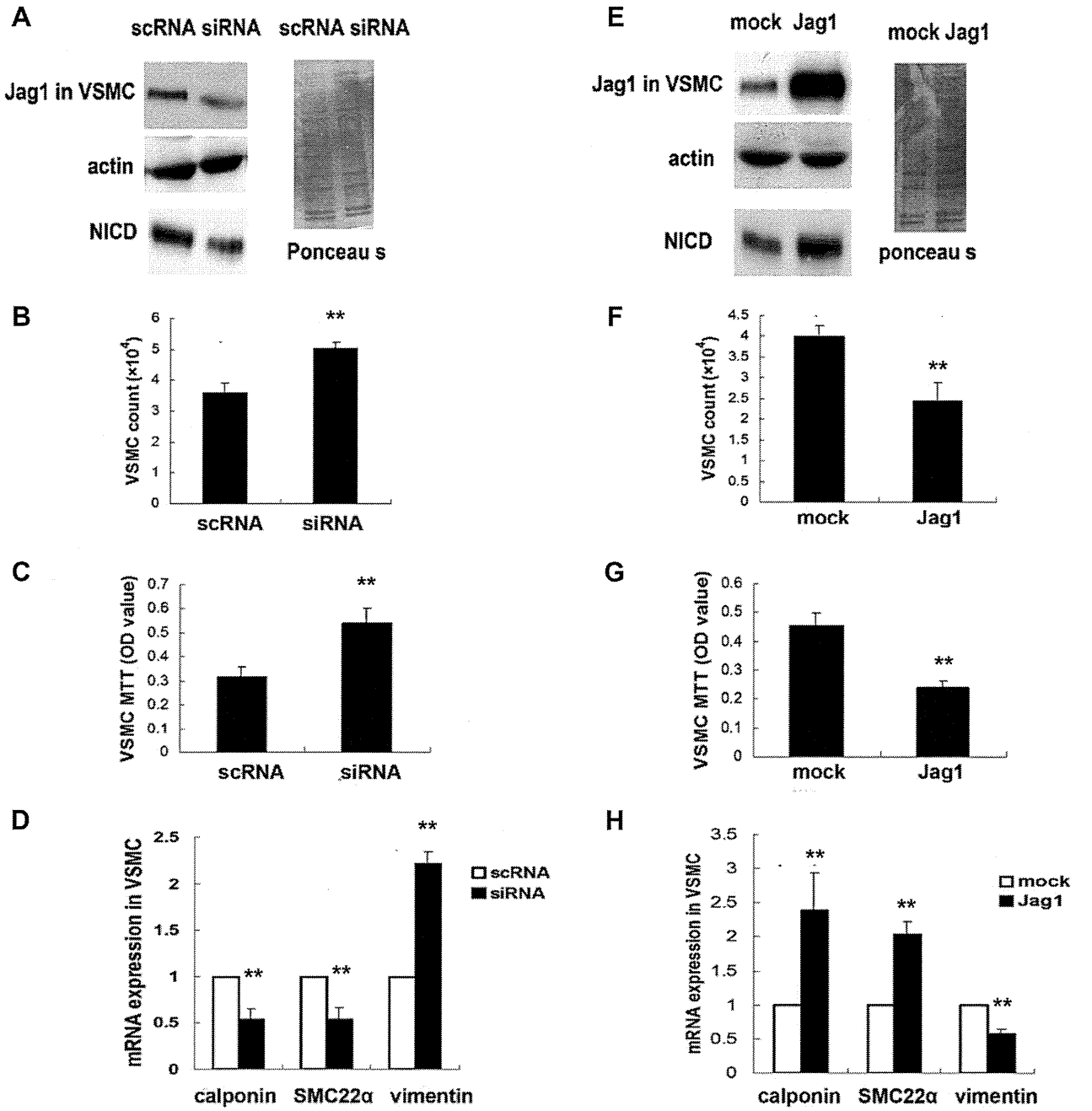



**Figure 3.** Jagged1 in vascular smooth muscle cells (VSMC) was sufficient to influence its synthetic transition. VSMC single culture experiments with small interfering RNA (siRNA) for Jagged1 or its control RNA (scRNA) (A through D), and with retrovirus encoding Jagged1 (Jag1) or its vector (mock) (E through H). A and E, Western blot analyses of Jagged1 from whole-cell lysates (actin served as loading control) and of Notch1 intracellular domain (NICD) from nuclear lysates (Ponceau S staining served as loading control). B and F, VSMC proliferation evaluated by cell counting after stimulation with 10% serum for 24 hours. C and G, VSMC proliferation evaluated by and MTT after stimulation with 10% serum for 24 hours. D and H, Messenger RNA expression of VSMC phenotype genes, including calponin, SM22$\alpha$ ,and vimentin analyzed by real-time PCR. $^{**}P<0.01$ versus scRNA or mock. n=3.

## Discussion

Many studies have demonstrated a link between aging and cell proliferation. In general, the decline of various cellular functions with aging is correlated with a decrease in the proliferative capacity of cells.[20] In the vascular bed, however, aging is associated with reduced EC regeneration but paradoxically with increased VSMC proliferation in vivo. It is still unclear how aging exerts a different effect on these 2 major types of vascular cells in the same arterial milieu. Among various factors that influence the functions of both EC and VSMC and are also related to the ageing process, Notch signaling is an important candidate. Because the most prominent Notch ligand and receptor are found to be Notch1 and Jagged1, we focused on these 2 molecules. We previously demonstrated that neointimal formation after vascular injury was exaggerated in aged rats and that the endothelial expression of Jagged1 after vascular injury decreased with aging.[11] However, the actual role of endothelial Jagged1 in

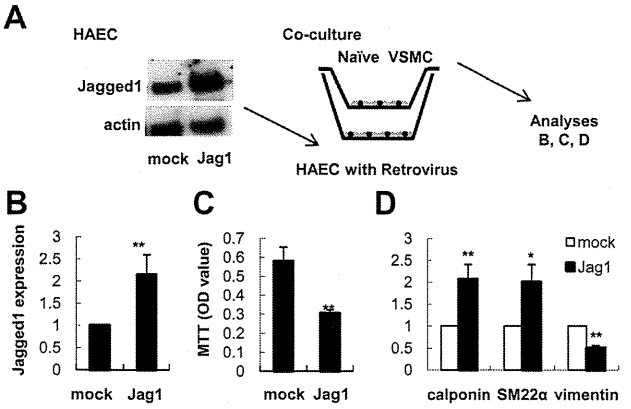



**Figure 4.** Overexpression of endothelial cell Jagged1 induced vascular smooth muscle cells (VSMC) to increase Jagged1 expression in company with contractile phenotype transition. A, Schematic model of the coculture and Jagged1 expression of human aortic endothelial cells (HAEC) after infection with Jagged1 retrovirus, shown by Western blot. B, Expression of Jagged1 in VSMC after coculture with Jagged1 overexpressing HAEC, quantified by real-time PCR. C, VSMC proliferation in response to 10% serum as evaluated by the MTT assay after coculture with Jagged1 overexpressing HAEC for 24 hours. D, Phenotype gene expression in VSMC by real-time PCR after coculture with Jagged1 overexpressing HAEC for 24 hours. $^{*}P<0.05$, $^{**}P<0.01$ versus mock. n=3.

vascular remodeling in vivo was unproven. In the present study, we established Tie2-cre$^{+}$ Jag1$^{lox/+}$ mice with EC showing heterozygous Jagged1 deficiency. Using these animals, we successfully demonstrated a causative role of endothelial Jagged1 in neointimal formation and medial thickening after vascular injury.

In models of vascular injury, the expression of Notch1, Notch3, and Jagged1 has been reported to show to be modulated.[7,21] There is a possibility that these other Notch ligands and receptors play some roles in our model. Nevertheless, we believe that Jagged1 and Notch1 play a prominent role because they are expressed predominantly in EC. It should also be noted that the Cre transgene of these mice was driven by the Tie-2 promoter, which is predominantly activated in endothelial cells but also in hematopoietic stem cells to some extent.[19] We found that peripheral blood cells from mutant mice did not differ from wild-type blood cells in the effect on VSMC phenotype transition in vitro. Nor could we find any influence of mutant bone marrow on arterial lesion formation in vivo. Thus, it is unlikely that heterozygous Jagged1 deficiency in blood cells contribute to enhanced formation of neointima in Tie2-cre$^{+}$ Jag1$^{lox/+}$ mice.

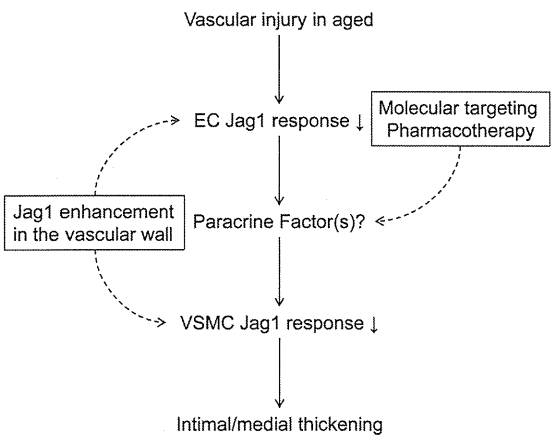



**Figure 5.** Jagged1 may be a novel therapeutic target for vascular remodeling after vascular injury. Vascular injury upregulates Jagged1 in endothelial cells (EC), which, through a putative paracrine effect, upregulates Jagged1 in vascular smooth muscle cells (VSMC). The increase of Jagged1 in VSMC induces its contractile transition, presumably through Notch signaling, which in turn might inhibit neointimal and medial thickening. Thus, a pharmacotherapy that enhances Jagged1 expression in vascular wall might be a novel strategy to prevent vascular remodeling associated with aging. The paracrine factor from EC that is responsible for the regulation of VSMC Jagged1 may also be used for this purpose.

The mechanism by which endothelial Jagged1 influences the phenotype of VSMC is another issue. High et al[8] reported that the primary role of endothelial Jagged1 is to potentiate the development of neighboring VSMC during embryonic vessel formation. Liu et al[22] found that EC Jagged1 induces mural cell differentiation through activation of Notch3 by using their in vitro system. Although Jagged1 is a major ligand of Notch, we suppose a paracrine mechanism rather than a direct cell-to-cell contact, as shown in these reports. In conduit arteries including the carotid arteries, EC and VSMC are separated by a thick basement membrane that is likely to inhibit direct contact of these cells. Our coculture model was also designed to prevent such contact, favoring a paracrine mechanism that influences VSMC behavior. Thus, in blood vessels, there may be 2 distinct mechanistic cascades governed by Jagged/Notch signaling, according to its anatomic or situational context.

Unexpectedly, we found in Tie2-cre$^{+}$ Jag1$^{lox/+}$ mice that the lack of Jagged1 expression by EC resulted in lack of Jagged1 expression by VSMC as well. This was also reproduced in the coculture model, suggesting that the effect results from EC/VSMC interaction. Moreover, in the monoculture experiment, deletion or introduction of Jagged1 was sufficient to influence the phenotypic transition of VSMC along with attenuation or enhancement of activated Notch1. Notch signaling has been reported to govern the differentiation and proliferation of VSMC.[23] Doi et al[24] reported that Jagged1/Notch signaling promotes VSMC differentiation. Also, Noseda et al[25] showed that the VSMC gene $\alpha$-SMA is a direct downstream target of Notch/CSL. Similar to these reports, our study confirmed negative regulation of the proliferation of VSMC by Jagged1. Taken together, it is likely that a paracrine factor or factors released from EC (driven by a Jagged1-dependent mechanism) influences the expression of Jagged1 by VSMC, which then regulates Notch signaling to finally drive the changes of VSMC phenotypic proteins.

Finally, we found that overexpression of Jagged1 by either EC or VSMC could prevent proliferation or synthesis by VSMC. We also found that Jagged1 expression by EC had a protective effect on EC themselves (X. Wu, K. Tateno, and T. Minamino, unpublished data). These findings strongly suggest that Jagged1 might act on both EC and VSMC to inhibit arterial lesion formation after vascular injury in vivo. Thus, it would be a plausible strategy to introduce a factor that enhances Jagged1 expression in the vascular wall to prevent

intimal/medial thickening in persons with ageing-related vascular diseases. Sethi et al[26] reported that in certain tumors, Jagged1 promotes its growth and metastasis. Thus, it should be noted with caution that enhancement of Jagged1 in vivo may potentially be hazardous in such situations. Another therapeutic approach would be to identify a Jagged1-driven paracrine factor that is secreted by EC and inhibits the overactivity of neighboring VSMC. Accordingly, it could be worthwhile to perform microarray analyses of samples derived from Jagged1 knockdown EC.

In conclusion, Jagged1 had a unidirectional effect on both EC and VSMC that inhibited arterial lesion formation after vascular injury. Our data support the possibility that Jagged1 may be a novel therapeutic target for aging-related vascular diseases.

## Sources of Funding

This work was supported by a grant-in-aid from National Natural Science Foundation of China (NSFC No. 30872710) to Dr Wu; a grant-in-Aid from National Natural Science Foundation of China (NSFC No. 30930043) and National Basic Research Program of China (2007CB512003) to Dr Zou; a grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture and Health and Labor Sciences Research Grants to Drs Komuro and Minamino. Dr Wu is also a recipient of grant from the Goho Life-Science in Japan.

## Disclosures

None.

## References

1. Mukamal KJ, Kronmal RA, Tracy RP, Cushman M, Siscovick DS. Traditional and novel risk factors in older adults: cardiovascular risk assessment late in life. *Am J Geriatr Cardiol.* 2004;13:69–80.
2. Najjar SS, Scuteri A, Lakatta EG. Arterial aging: is it an immutable cardiovascular risk factor? *Hypertension.* 2005;46:454–462.
3. Yildiz O. Vascular smooth muscle and endothelial functions in aging. *Ann N Y Acad Sci.* 2007;1100:353–360.
4. Losordo DW, Isner JM, Diaz-Sandoval LJ. Endothelial recovery: the next target in restenosis prevention. *Circulation.* 2003;107:2635–2637.
5. Carey KA, Farnfield MM, Tarquinio SD, Cameron-Smith D. Impaired expression of notch signaling genes in aged human skeletal muscle. *J Gerontol A Biol Sci Med Sci.* 2007;62:9–17.
6. Conboy IM, Conboy MJ, Smythe GM, Rando TA. Notch-mediated restoration of regenerative potential to aged muscle. *Science.* 2003;302:1575–1577.
7. Gridley T. Notch signaling in vascular development and physiology. *Development.* 2007;134:2709–2718.
8. High FA, Lu MM, Pear WS, Loomes KM, Kaestner KH, Epstein JA. Endothelial expression of the notch ligand jagged1 is required for vascular smooth muscle development. *Proc Natl Acad Sci U S A.* 2008;105:1955–1959.
9. Li Y, Takeshita K, Liu PY, Satoh M, Oyama N, Mukai Y, Chin MT, Krebs L, Kotlikoff MI, Radtke F, Gridley T, Liao JK. Smooth muscle notch1 mediates neointimal formation after vascular injury. *Circulation.* 2009;119:2686–2692.
10. Suchting S, Eichmann A. Jagged gives endothelial tip cells an edge. *Cell.* 2009;137:988–990.
11. Wu X, Zhou Q, Huang L, Sun A, Wang K, Zou Y, Ge J. Ageing-exaggerated proliferation of vascular smooth muscle cells is related to attenuation of jagged1 expression in endothelial cells. *Cardiovasc Res.* 2008;77:800–808.
12. Mancini SJ, Mantei N, Dumortier A, Suter U, MacDonald HR, Radtke F. Jagged1-dependent notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood.* 2005;105:2340–2342.
13. Tateno K, Minamino T, Toko H, Akazawa H, Shimizu N, Takeda S, Kunieda T, Miyauchi H, Oyama T, Matsuura K, Nishi J, Kobayashi Y, Nagai T, Kuwabara Y, Iwakura Y, Nomura F, Saito Y, Komuro I. Critical roles of muscle-secreted angiogenic factors in therapeutic neovascularization. *Circ Res.* 2006;98:1194–1202.
14. Kumar A, Lindner V. Remodeling with neointima formation in the mouse carotid artery after cessation of blood flow. *Arterioscler Thromb Vasc Biol.* 1997;17:2238–2244.
15. Mukai Y, Rikitake Y, Shiojima I, Wolfrum S, Satoh M, Takeshita K, Hiroi Y, Salomone S, Kim HH, Benjamin LE, Walsh K, Liao JK. Decreased vascular lesion formation in mice with inducible endothelial-specific expression of protein kinase akt. *J Clin Invest.* 2006;116:334–343.
16. Li J, Garnette CS, Cahn M, Claytor RB, Rohrer MJ, Dobson JG Jr, Gerlitz B, Cutler BS. Recombinant thrombomodulin inhibits arterial smooth muscle cell proliferation induced by thrombin. *J Vasc Surg.* 2000;32:804–813.
17. Miyauchi H, Minamino T, Tateno K, Kunieda T, Toko H, Komuro I. Akt negatively regulates the in vitro lifespan of human endothelial cells via a p53/p21-dependent pathway. *EMBO J.* 2004;23:212–220.
18. Sakamoto K, Ohara O, Takagi M, Takeda S, Katsube K. Intracellular cell-autonomous association of notch and its ligands: a novel mechanism of notch signal modification. *Dev Biol.* 2002;241:313–326.
19. Tang Y, Harrington A, Yang X, Friesel RE, Liaw L. The contribution of the tie2+ lineage to primitive and definitive hematopoietic cells. *Genesis.* 2010;48:563–567.
20. Carlson ME, Silva HS, Conboy IM. Aging of signal transduction pathways, and pathology. *Exp Cell Res.* 2008;314:1951–1961.
21. Lindner V, Booth C, Prudovsky I, Small D, Maciag T, Liaw L. Members of the jagged/notch gene families are expressed in injured arteries and regulate cell phenotype via alterations in cell matrix and cell-cell interaction. *Am J Pathol.* 2001;159:875–883.
22. Liu H, Kennard S, Lilly B. Notch3 expression is induced in mural cells through an autoregulatory loop that requires endothelial-expressed jagged1. *Circ Res.* 2009;104:466–475.
23. Morrow D, Guha S, Sweeney C, Birney Y, Walshe T, O'Brien C, Walls D, Redmond EM, Cahill PA. Notch and vascular smooth muscle cell phenotype. *Circ Res.* 2008;103:1370–1382.
24. Doi H, Iso T, Sato H, Yamazaki M, Matsui H, Tanaka T, Manabe I, Arai M, Nagai R, Kurabayashi M. Jagged1-selective notch signaling induces smooth muscle differentiation via a rbp-jkappa-dependent pathway. *J Biol Chem.* 2006;281:28555–28564.
25. Noseda M, Fu Y, Niessen K, Wong F, Chang L, McLean G, Karsan A. Smooth muscle alpha-actin is a direct target of notch/csl. *Circ Res.* 2006;98:1468–1470.
26. Sethi N, Dai X, Winter CG, Kang Y. Tumor-derived jagged1 promotes osteolytic bone metastasis of breast cancer by engaging notch signaling in bone cells. *Cancer Cell.* 2011;19:192–205.

# Supplemental Materials

## Supplemental methods

### Quantitative Real-time Reverse-Transcription Polymerase Chain Reaction

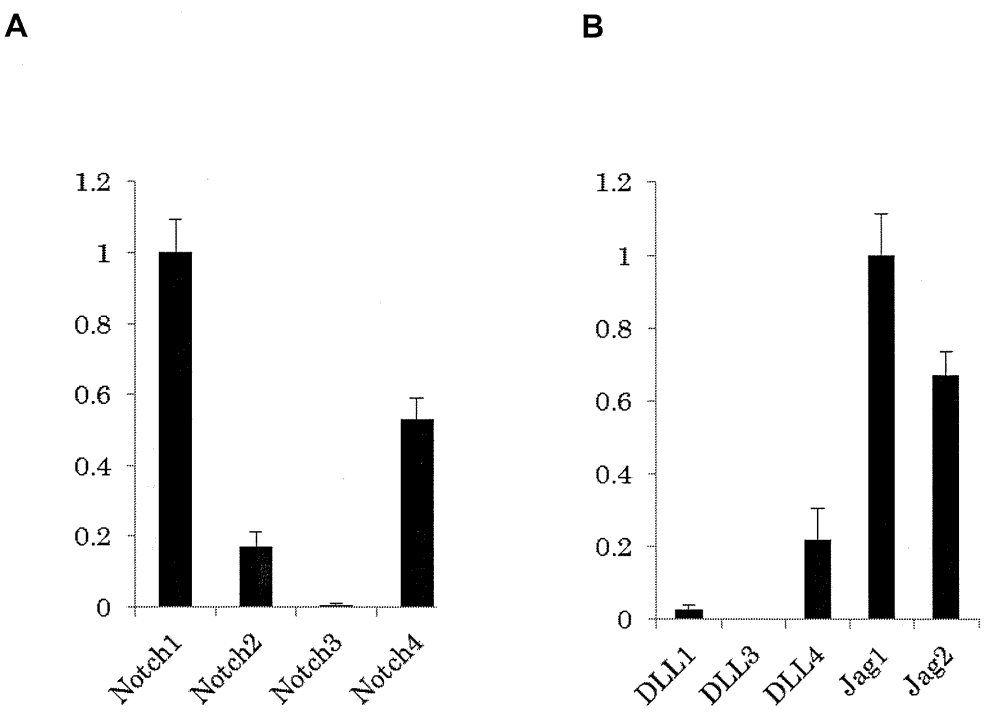
RNA was isolated from carotid artery or cultured cells using the RNeasy mini kit (Qiagen), and then the extract RNA was reverse transcribed by QuantiTect Reverse Transcription kit (Qiagen). Amplification was performed with a LightCycler® 480 (Roche Diagnostic Ltd) according to the manufacturer's instructions. Sequences of the primers and number of the probe (Universal probelibrary set, Roche, Indianapolic, IN, USA) are indicated (supplement Tab). Relative mRNA expression was calculated with the comparative $C_T$ method and normalized to the expression of the endogenous 18s and GAPDH.

**Supplemental Table**

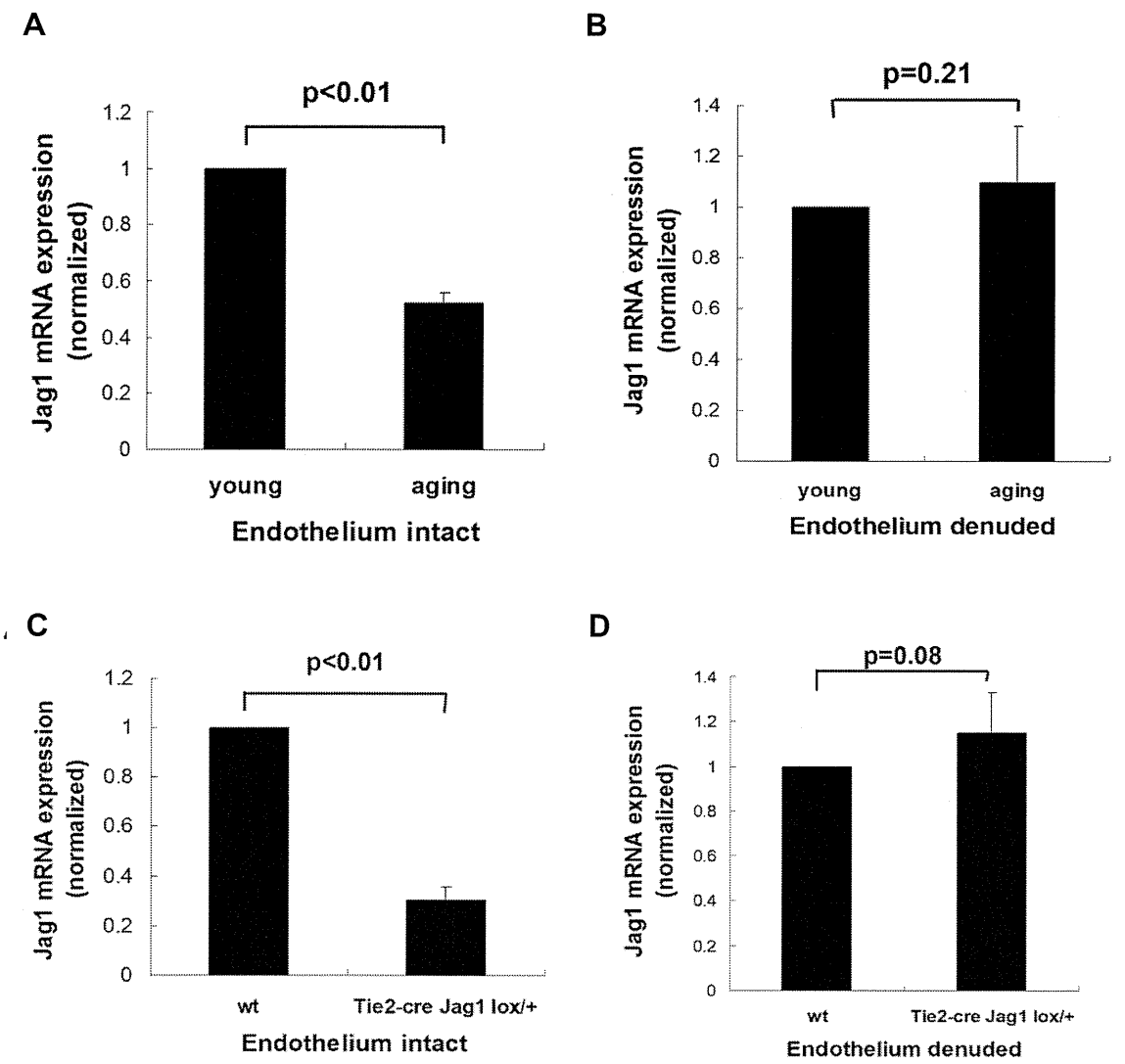
**Primers and conditions for real-time PCR**

| Gene | Forward primer 5'-3' | Reverse primer 5'-3' | Probe number |
|---|---|---|---|
| Mouse 18s | tggcaaacttgccttatcaga | gaagagcaaactctctctgaaacata | #55 |
| Mouse Jagged1 | gaggcgtcctctgaaaaaca | acccaagccactgttaagaca | #6 |
| Human GAPDH | agccacatcgctcagacac | gcccaatacgaccaaatcc | #60 |
| Human Jagged1 | gaatggcaacaaaacttgcat | agccttgtcggcaaatagc | #42 |
| Human P16 | Gtggacctggctgaggag | Ctttcaatcggggatgtctg | #34 |
| Human P21 | Cgaagtcagttccttgtggag | Catgggttctgacggacat | #82 |
| Human calponin | ccaaccatacacaggtgcag | tcaccttgtttcctttcgtctt | #79 |
| Human SM22α | aggccaagattgcacagc | ccgcctgtttctctctgg | #68 |
| Human vimentin | aaagtgtggctgccaagaac | agcctcagagaggtcagcaa | #16 |

**Supplemental Figure I. Expression of Notch receptors and ligands.** Relative copy numbers of Notch receptors (A) and ligands (B) quantified by real-time PCR



**Supplemental Figure II. Expression of Jagged1 in the carotid artery.** A and B, Jagged1 expression of carotid artery wall of young and aged mice quantified by real-time PCR. Endothelial monolayer was left intact (A), or denuded with a scraper (B). C and D, Jagged1 expression of carotid artery wall of Tie2-cre Jag1$^{lox/+}$ mice. Endothelial monolayer was left intact (C), or denuded with a scraper (D).

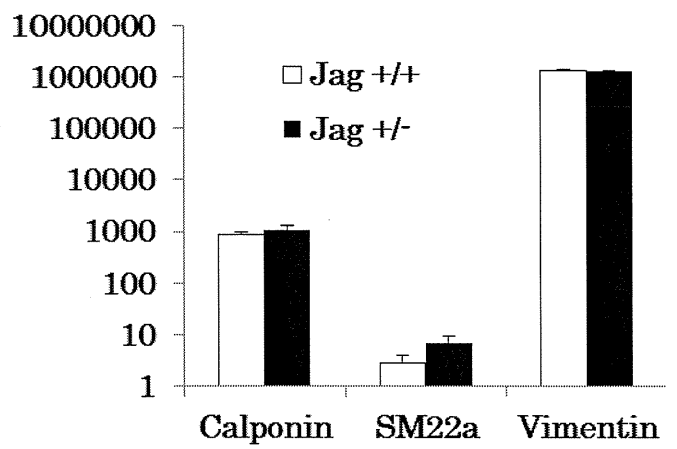


**Supplemental Figure III. Significance of bone marrow derived cells from the Tie2-cre Jag1$^{lox/+}$ mice.** A, Blood cell count of adult Jagged1 hetero-deficient mice. Data indicate mean ± SE. B and C, Co-culture of VSMC with peripheral blood cells from Jagged1 hetero-deficient mice. Phenotypic gene expression in VSMC after co-culture, quantified by real-time PCR (B). Note that PCR value is in logarithm. Expression of Jagged1 in VSMC after co-culture (C). D and E, Neointimal area (D) and I/M ratio (E) following carotid artery ligation of wild type mice, in which bone marrow was replaced with the one from Jagged1 hetero-deficient mice.

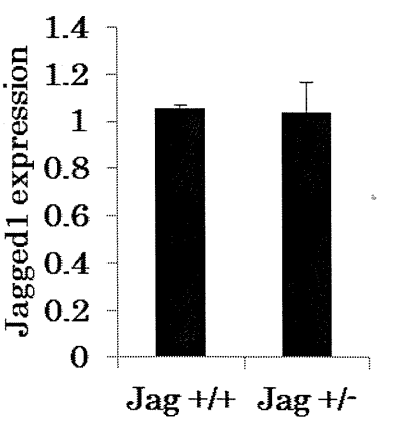
**A.**

| | WBC /uL | Seg (%) | Mono (%) | Lymph (%) | Hb g/dL | MCV u3 | Plt x10E4 |
|---|---|---|---|---|---|---|---|
| WT | 5057±756 | 14.2±1.8 | 1.6±0.2 | 84.0±1.9 | 13.6±0.3 | 54.0±1.0 | 91.2±2.4 |
| KO | 6325±1232 | 10.5±2.5 | 1.5±0.5 | 87.5±2.5 | 14.0±0.8 | 55.3±0.9 | 68.5±8.5 |

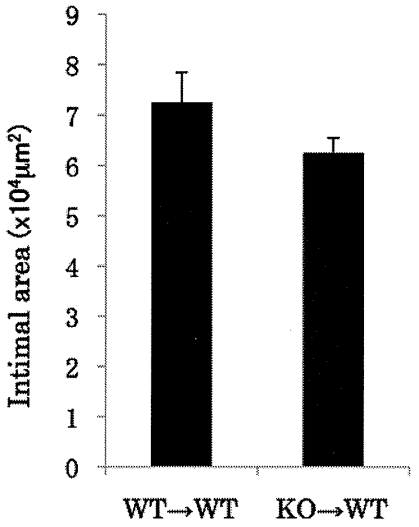
**B**



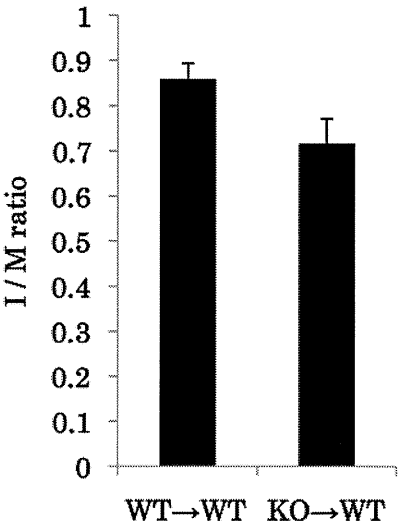

**C**



D




E



Atherosclerosis 218 (2011) 226–232

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

# Serum apolipoprotein B-48 levels are correlated with carotid intima-media thickness in subjects with normal serum triglyceride levels

Kazuhiro Nakatani[a,1], Taizo Sugimoto[b,2], Daisaku Masuda[a,*], Rieko Okano[c,3], Tomoko Oya[c,3], Yasumasa Monden[c,3], Taiji Yamashita[a,1], Ryota Kawase[a,1], Hajime Nakaoka[a,1], Miwako Inagaki[a,1], Miyako Yuasa-Kawase[a,1], Kazumi Tsubakio-Yamamoto[a,1], Tohru Ohama[a,1], Makoto Nishida[a,d,1,4], Masato Ishigami[a,e,1,5], Issei Komuro[a,1], Shizuya Yamashita[a,1]

[a] *Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*
[b] *Sugimoto Clinic, 2-21-18 Momodani, Ikuno, Osaka 544-0034, Japan*
[c] *Osaka Police Hospital Dock, 15-15 Ishigatsuji, Tennouji, Osaka 543-0031, Japan*
[d] *Health Care Center, Osaka University, 1-7 Machikaneyama, Toyonaka, Osaka 560-0043, Japan*
[e] *Division of Health Sciences, Osaka University Graduate School of Medicine, 1-7 Yamadaoka, Suita, Osaka 565-0871, Japan*

ARTICLE INFO

*Article history:*
Received 13 September 2010
Received in revised form 30 April 2011
Accepted 11 May 2011
Available online 18 May 2011

*Keywords:*
Apolipoprotein B-48
Atherosclerosis
Carotid intima-media thickness
Chylomicron remnants
Postprandial hyperlipidemia

ABSTRACT

*Background:* Postprandial hyperlipidemia (PPHL) is an independent risk factor for coronary heart disease (CHD) which is based on the accumulation of chylomicrons (CM) and CM remnants containing apolipoprotein B-48 (apoB-48). Since atherosclerotic cardiovascular diseases are frequently observed even in subjects with normal serum triglyceride (TG) level, the correlation between fasting apoB-48 containing lipoproteins and carotid intima-media thickness (IMT) was analyzed in subjects with normal TG levels.
*Methods:* From subjects who took their annual health check at the Osaka Police Hospital ($n = 245$, male), one-hundred and sixty-four male subjects were selected to take part in this study; the excluding factors were: systolic blood pressure $\geq$140 mmHg, intake of antihypertensive or antihyperlipidemic drugs, or age >65 years. The association between biochemical markers and IMT was analyzed and independent predictors of max-IMT were determined by multiple regression analysis in all subjects and in groups N-1 (TG < 100 mg/dl, $n = 58$), N-2 (100 $\leq$ TG < 150 mg/dl, $n = 53$) and H (150 $\leq$ TG mg/dl, $n = 53$), respectively.
*Results:* Fasting total cholesterol, LDL–cholesterol, HDL–cholesterol, apoB-100 and ln RemL–C (remnant lipoprotein–cholesterol) levels were not correlated with max-IMT, but ln TG and ln apoB-48 were significantly correlated with max-IMT in all subjects. Ln apoB-48 and apoB-48/TG ratio were significantly correlated with max-IMT in group N-2. By multiple regression analysis, age and ln apoB-48 were independent variables associated with max-IMT in group N-2.
*Conclusion:* Serum apoB-48 level might be a good marker for the detection of early atherosclerosis in middle-aged subjects with normal-range levels of blood pressure and TG.


## 1. Introduction

Hypercholesterolemia, including high serum LDL-cholesterol (LDL-C) level, is strongly correlated to the development of atherosclerotic cardiovascular diseases [1]. Statins significantly decrease LDL-C levels and the morbidity of atherosclerotic cardiovascular diseases; however, they cannot completely prevent the occurrence of these diseases yet [2]. Epidemiologic studies have revealed that fasting hypertriglyceridemia is also associated with atherosclerosis, independent of other coronary risk factors such as high LDL-C level [3,4]. A case-control study showed that fasting and non-fasting TG levels were also superior among patients with coronary heart disease (CHD) as compared with control subjects [5]. A Japanese prospective study demonstrated that not only fast-

*Abbreviations:* BMI, body mass index; apoB-48, apolipoprotein B-48; PPHL, postprandial hyperlipidemia; CM, chylomicrons; CMR, chylomicron remnants; RemL-C, remnant lipoprotein-cholesterol; TG, triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; HOMA-IR, homeostasis model assessment as an index of insulin resistance; IRI, immuno-reactive insulin; IMT, intima-media thickness.

* Corresponding author. Tel.: +81 6 6879 3633; fax: +81 6 6879 3645.
*E-mail address:* masuda@imed2.med.osaka-u.ac.jp (D. Masuda).
[1] Tel.: +81 6 6879 3633; fax: +81 6 6879 3645.
[2] Tel.: +81 6 6716 4910; fax: +81 6 6715 1724.
[3] Tel.: +81 6 6775 3131; fax: +81 6 6775 3121.
[4] Tel.: +81 6 6850 6015; fax: +81 6 6850 6040.
[5] Tel.: +81 6 6879 2588; fax: +81 6 6879 2499.

0021-9150/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2011.05.009

ing but also non-fasting TG levels were significantly correlated with CHD morbidity [6]. In this study, the authors also showed that an increase in TG levels was significantly correlated with an increase in CHD morbidity even though TG levels remained below 150 mg/dl, a level which has been recognized as borderline of high risk status for atherosclerotic cardiovascular diseases on the basis of Framingham Study [7]. Therefore, we need to evaluate the emerging risk of atherosclerotic cardiovascular diseases even in subjects with normotriglyceridemia (TG < 150 mg/dl).

Postprandial hyperlipidemia (PPHL) is caused by the impaired metabolism of lipoproteins, which is mainly characterized by a postprandial accumulation of intestine-derived lipoproteins, chylomicrons (CM) and their hydrolyzed lipoproteins, chylomicron remnants (CM-R). In subjects with normal lipoprotein metabolism, CM and CM-R are promptly hydrolyzed, diminished in size and cleared from the circulation by the liver within a few hours after a meal. PPHL does not indicate the postprandial increase of lipids and lipoproteins which are promptly cleared from the circulation in subjects with normal lipoprotein metabolism. However, in patients with PPHL, CM-R continue to accumulate for over 6–8 h after a meal, penetrating into the vessels to form foam cells. Many recent studies have proved that PPHL is an independent risk factor for the development of CHD and atherosclerosis of carotid arteries [8–10]. Many basic studies have suggested that accumulated CM-R particles may promote atherogenicity in the arterial wall [11]. An oral fat loading (OFL) test is sometimes used to assess PPHL levels; however, this it is not a suitable testing option for routine clinical use because it requires a lot of time (6–8 h). Further, consensus has not yet been reached regarding the indication and the interpretation of data from this test. We developed a novel enzyme-linked immuno-sorbent assay (ELISA) to measure serum levels of apolipoprotein B-48 (apoB-48) [12]. Since one apoB-48 molecule is included in one CM and CM-R particle up to the clearance by the liver, serum apoB-48 level represents the number of both CM and CM-R particles and is suitable for the quantitative evaluation of postprandial changes. In patients with suspected accumulation of CM and CM-R, serum apoB-48 levels are significantly higher at the fasting state and increased after OFL in normolipidemic subjects [12]. High levels of fasting serum apoB-48 suggest the existence of PPHL, without performing an OFL test [13], and are reportedly related to the development of atherosclerotic cardiovascular diseases [14–17]. These results suggest that fasting apoB-48 level is a good marker for the evaluation of atherogenic risk in patients with hypertriglyceridemia. However, very few studies have so far investigated the correlation between fasting serum apoB-48 levels and the development of atherosclerosis among subjects with normal fasting TG levels.

In the current study, we have investigated the correlations between profiles of apoB-48-containing lipoproteins and the progression of atherosclerosis in subjects with normal TG levels. For the evaluation of atherosclerosis progression, intima-media thickness (IMT) of carotid arteries was measured using a diagnostic ultrasound, which was shown to be significantly correlated with the development and prognosis of CHD and cerebrovascular diseases [18,19].

## 2. Subjects and methods

### 2.1. Subjects

A consecutive series of subjects ($n = 245$, male) who came to Osaka Police Hospital for the annual health checkup were picked up serially. One-hundred and sixty-four male subjects were finally enrolled by the following exclusion criteria: systolic blood pressure $\geq$140 mmHg, age over 65 years and intake of any drugs affecting lipid metabolism and blood pressure. This study was approved by the Ethical Committee of Osaka Police Hospital, and all participants gave their written informed consent.

### 2.2. Biochemical analyses

Height, weight, and waist circumference were measured in the standing position. Systolic and diastolic blood pressures were measured at rest in the sitting position. Blood samples were collected after an overnight fast, followed by an immediate separation of serum and plasma. Total cholesterol (TC), triglycerides (TG), HDL–C, fasting plasma glucose (FPG) and uric acid (UA) levels were measured by enzymatic methods, LDL–C levels by direct method, and serum apoB levels by immunoturbidity method, respectively (Sekisui Medical Co., Ltd., Tokyo, Japan). Hemoglobin A1c (HbA1c) levels were determined by high performance liquid chromatography (HPLC) method and immunoreactive insulin (IRI) levels by the immunoturbidity method (SRL Inc., Tokyo, Japan). Serum apoB-48 levels were measured by the chemiluminescent enzyme immunoassay (CLEIA) using anti-human apoB-48 monoclonal antibodies, which we developed previously with minor modification (Fujirebio Inc., Tokyo, Japan). Remnant lipoprotein–cholesterol (RemL–C) levels were measured by the homogenous assay (Kyowa Medex, Tokyo, Japan) [12]. ApoB-100 levels were calculated by subtracting the value of apoB-48 from the value of serum apoB. Plasma adiponectin levels were determined by the human adiponectin ELISA kit (Otsuka Pharmaceuticals, Tokyo, Japan). Subjects were divided into 3 groups by serum TG level: group N-1 ($n = 58$), TG < 100 mg/dl; Group N-2 ($n = 53$), 100 $\leq$ TG < 150 mg/dl and Group H ($n = 53$), 150 $\leq$ TG mg/dl.

### 2.3. Ultrasound measurements

The IMT of carotid arteries was determined using ultrasonography in the supine position. High-resolution B-mode ultrasound images were obtained (Toshiba Nemio, Toshiba Corp., Tokyo, Japan) with a 12 MHz linear array transducer. Three arterial wall segments in each carotid artery were imaged from a fixed lateral transducer angle at the far wall. All segments, including both sides of common carotid artery, the carotid bifurcation, and the internal carotid artery, were scanned. The thickest part of the IMT was recorded as max-IMT, and the IMT of the far wall was measured at 3 continuous sites at a 1.0-cm interval proximal to the thickest part of IMT in each side and then averaged to obtain mean-IMT. The mean-IMT value and greater max-IMT value obtained from scans of the right and left carotid arteries in each subject were used for statistical analyses.

### 2.4. Statistical analysis

Values were expressed as mean $\pm$ SD. ApoB-48 levels were normalized by logarithmic transformation. Between-group comparisons of the means and median were performed by Tukey's HSD test among group N-1, group N-2 and group H. The correlations between metabolic parameters and mean-/max-IMT were calculated by Pearson's correlation coefficients. Stepwise multiple regression analysis was used to determine independent predictors of max-IMT measurement with $P$ value-to-enter set at 0.20. Age, sBP, dBP, total cholesterol, ln TG, LDL–C, HDL–C, apoB-48, apoB-100, ln RemL–C, FPG, HbA1c, ln HOMA-IR, and IRI were included as explanatory variables in the method. Data were analyzed with JMP8 software (SAS Institute, Cary, NC). All statistical significance was accepted at $P < 0.05$.

**Table 1**
Clinical profiles of subjects investigated.

| | Total<br>$n=164$ | Group N-1<br>TG < 100<br>$n=58$ | Group N-2<br>100 ≤ TG < 150<br>$n=53$ | Group H<br>150 ≤ TG<br>$n=53$ |
|---|---|---|---|---|
| Age (year) | 52 ± 6 | 53 ± 6 | 52 ± 6 | 52 ± 7 |
| BMI (kg/m2) | 24.7 ± 3.0 | 23.4 ± 2.3 | 24.6 ± 2.5 | 26.1 ± 3.4$^{\#}$ |
| Waist circ. (cm) | 87 ± 8 | 83 ± 6 | 88 ± 7$^{**}$ | 91 ± 8$^{\#}$ |
| sBP (mmHg) | 120 ± 12 | 117 ± 12 | 120 ± 11 | 123 ± 12 |
| dBP (mmHg) | 82 ± 9 | 79 ± 9 | 82 ± 9 | 84 ± 9 |
| TC (mg/dl) | 208 ± 30 | 201 ± 27 | 211 ± 30 | 213 ± 32 |
| HDL-C (mg/dl) | 54 ± 13 | 60 ± 14 | 56 ± 11 | 47 ± 8$^{\#\#}$ |
| LDL-C (mg/dl) | 124 ± 28 | 123 ± 24 | 129 ± 26 | 119 ± 33 |
| TG (mg/dl) | 152 ± 120 | 77 ± 15 | 122 ± 15$^{*}$ | 264 ± 156$^{\#\#}$ |
| apoB-48 (mg/dl) | 0.57 ± 0.55 | 0.28 ± 0.14 | 0.42 ± 0.19 | 1.03 ± 0.74$^{\#\#}$ |
| apoB-100 (mg/dl) | 97.8 ± 17.3 | 89.6 ± 15.1 | 100.8 ± 14.6$^{*}$ | 103.7 ± 18.8 |
| RemL-C (mg/dl) | 12.2 ± 8.3 | 7.0 ± 5.0 | 9.5 ± 2.1 | 20.4 ± 8.9$^{\#\#}$ |
| FPG (mg/dl) | 96 ± 14 | 96 ± 13 | 98 ± 15 | 95 ± 15 |
| HbA1c (%) | 5.1 ± 0.5 | 5.1 ± 0.5 | 5.2 ± 0.4 | 5.1 ± 0.6 |
| HOMA-IR | 1.3 ± 0.9 | 1.0 ± 0.5 | 1.3 ± 0.8 | 1.6 ± 1.1 |
| IRI (μU/ml) | 5.2 ± 2.9 | 4.0 ± 1.9 | 5.1 ± 3.0 | 6.5 ± 3.4$^{\#}$ |
| Adiponectin (μg/ml) | 5.4 ± 3.1 | 6.4 ± 3.9 | 5.2 ± 2.5 | 4.5 ± 2.3 |

From male subjects who took their annual health checkup at Osaka Police Hospital, one-hundred and sixty-four male subjects (aged 52 ± 6 years) were divided into 3 groups by serum TG level; group N-1 ($n=58$), TG < 100 mg/dl; group N-2 ($n=53$), 100 ≤ TG < 150 mg/dl; group H ($n=53$), 150 ≤ TG mg/dl, respectively. Values are the mean ± SD; between-group comparisons of the means and median were performed by Tukey's HSD test among group N-1, group N-2 and group H.

$^{*}$ $P<0.05$ (group N-2 compared with group N-1).
$^{**}$ $P<0.005$ (group N-2 compared with group N-1).
$^{\#}$ $P<0.05$ (group H compared with group N-2).
$^{\#\#}$ $P<0.005$ (group H compared with group N-2).

## 3. Results

### 3.1. Clinical profiles

Table 1 shows the clinical profiles of all patients ($n=164$), group N-1 ($n=58$, TG < 100 mg/dl), group N-2 ($n=53$, 100 ≤ TG < 150 mg/dl) and group H ($n=53$, 150 ≤ TG mg/dl). The subjects were 52 ± 6 years-old (mean ± SD), and apoB-48 level was 0.57 ± 0.55 mg/dl. Waist circumference, TG and apoB-100 levels in group N-2 were significantly higher than those of group N-1. BMI, waist circumference, TG, apoB-48, apoB-100 and RemL-C levels in group H were significantly higher, and HDL-C levels were significantly lower in group H than in group N-2. Mean- and max-IMT were measured in all subjects, and between-group comparisons of the means and median were performed by Tukey's HSD test among total subjects, group N-1, group N-2 and group H. There was no significant difference in mean-IMT (total subjects, 0.80 ± 0.18 mm; group N-1, 0.75 ± 0.13 mm; group N-2, 0.79 ± 0.17 mm; and group H, 0.84 ± 0.23 mm, respectively) and in max-IMT (total subjects, 0.87 ± 0.23 mm; group N-1, 0.81 ± 0.16 mm; group N-2, 0.86 ± 0.22 mm; and group H, 0.93 ± 0.29 mm, respectively).

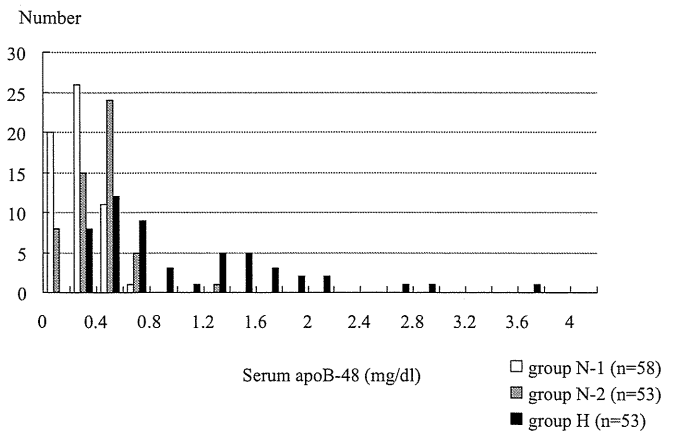



**Fig. 1.** Distribution of fasting serum apoB-48 levels. Geometric means were 0.24 mg/dl in group N-1, 0.41 mg/dl in group N-2 and 0.69 mg/dl in group H. The distribution of apoB-48 levels was significantly shifted to higher values; the data was normalized by logarithmic transformation for further statistical analysis.

### 3.2. Distribution of apoB-48 in each TG group

For the analysis of the correlation between apoB-48 levels and IMT, the distribution of apoB-48 levels was compared among groups N-1, N-2 and H (Fig. 1). The distribution of apoB-48 levels in group H was significantly shifted to higher values as compared with group N-1 and group N-2. ApoB-48 levels in group N-2 were also shifted to higher values compared with group N-1. In order to compare the apoB-48 levels in these TG groups, we normalized the apoB-48 levels by logarithmic transformation for further statistical analysis.

### 3.3. Correlation analysis in all subjects with max-IMT

Coronary risk factors such as TC, LDL-C, HDL-C, apoB-100 and ln RemL-C levels showed no significant correlations with mean- and max-IMT as assessed by Pearson's correlation coefficients in total subjects. To the contrary, ln TG and ln apoB-48 levels were significantly correlated with max-IMT (Fig. 2), and not significantly correlated with mean-IMT levels.

### 3.4. Correlation analysis in each TG group with max-IMT

The correlation of fasting apoB-100 levels and ln apoB-48 levels was analyzed with max-IMT in groups N-1, N-2 and H, respectively (Fig. 3). ApoB-100 levels were not significantly correlated with max-IMT in each TG group. Ln apoB-48 levels were significantly correlated with max-IMT only in group N-2.

### 3.5. Correlation analysis of apoB-48/TG ratio in each TG group with max-IMT

The significant correlation between ln apoB-48 levels and max-IMT means that the increase in apoB-48-containing lipoproteins might promote atherosclerosis in the carotid artery. The correla-

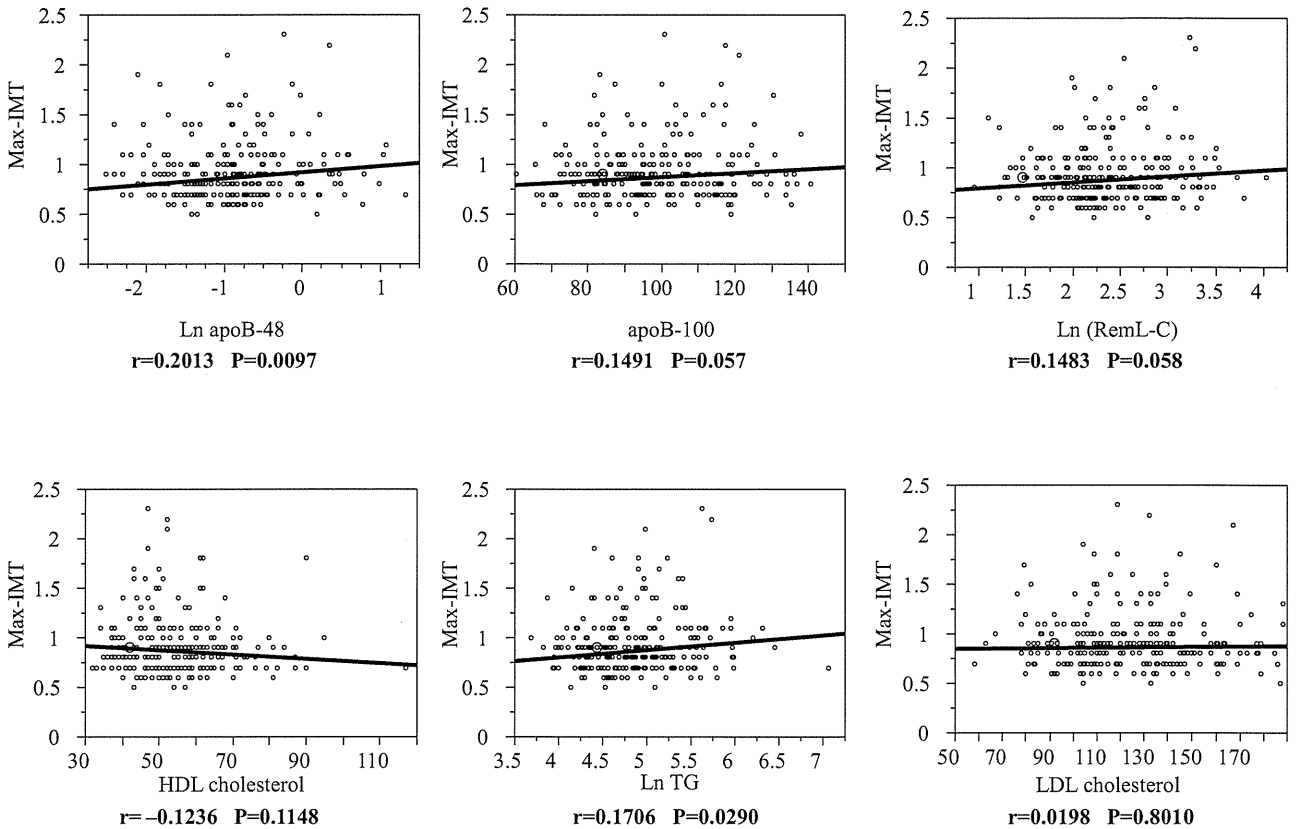



**Fig. 2.** Correlations between max-IMT and fasting lipid profiles. Because the distribution of apoB-48, TG and RemL–C was skewed to the left, the data were normalized by logarithmic transformation for statistical analysis. The fasting serum concentrations of TC, LDL–C, HDL–C, apoB-100 and ln RemL–C were not significantly correlated with max-IMT, but ln TG and ln apoB-48 were significantly correlated with max-IMT. The correlations were calculated by Pearson's correlation coefficients, and statistical significance was accepted at $P < 0.05$.

tions with max-IMT of fasting apoB-48/TG ratio, which refers to the number of CM-R lipoprotein particles, were evaluated and shown to be significant in group N-2, but not in N-1 and H (Fig. 4).

### *3.6. Stepwise multiple regression analysis between max-IMT and biochemical parameters*

By multiple regression analysis, the correlations between max-IMT and age, blood pressure, lipid profiles and glucose-related parameters were assessed. Age, systolic blood pressure (sBP), diastolic blood pressure (dBP), TC, ln TG, LDL–C, HDL–C, apoB-48, apoB-100, ln RemL–C, FPG, HbA1c, ln HOMA-IR, and IRI were independent variables. Among these parameters, age, sBP and ln apoB-48 were independent variables associated with max-IMT level in all subjects (Table 2). In group N-2, age and ln apoB-48 were independent variables associated with max-IMT, but sBP was not. HbA1c was an independent variable associated with max-IMT in group N-1.

## 4. Discussion

A positive correlation between fasting serum apoB-48 levels and IMT was observed in patients with hypertriglyceridemia or diabetes mellitus [14,17]. Significantly high TG level (TG >150 mg/dl) is correlated to an impaired metabolism of TG-rich lipoproteins in endogenous (VLDL and LDL) and exogenous (CM and CM-R) lipoprotein pathways, which are strongly related to the development of atherosclerosis and the morbidity of cardiovascular diseases. In the current study, our results showed that fasting serum apoB-48 levels are correlated with max-IMT in subjects with relatively high, but normal TG level (from 100 to 150 mg/dl).

### *4.1. Contribution of increased CM-R to atherosclerosis*

A postprandial increase in remnants has been considered as atherogenic since Zilversmit proposed his postprandial hyperlipidemia concept over 30 years ago [20]. Several studies indicate that apoB-48-containing lipoproteins have various kinds of atherogenic

**Table 2**
Stepwise multiple regression analysis of max-IMT in relation to age, blood pressure, lipid profiles, and glucose-related parameters.

| | All subjects | | Group (N-1) | | Group (N-2) | | Group (H) | |
|---|---|---|---|---|---|---|---|---|
| | *F* value | *P* value | *F* value | *P* value | *F* value | *P* value | *F* value | *P* value |
| Age | 18.889 | <0.0001 | Not remain | | 5.51 | 0.023 | 12.603 | 0.0009 |
| sBP | 6.467 | 0.0120 | Not remain | | Not remain | | 8.249 | 0.0060 |
| ln apoB-48 | 5.542 | 0.0198 | Not remain | | 5.106 | 0.0283 | Not remain | |
| HbA1c | 2.541 | 0.1129 | 6.123 | 0.0164 | 2.098 | 0.1538 | Not remain | |

Stepwise multiple regression analysis was used to determine independent predictors of max-IMT measurement with *P* value-to-enter and *P* value-to-retain set at 0.20. Age, sBP, dBP, TC, ln TG, LDL–C, HDL–C, apoB-48, apoB-100, ln RemL–C, FPG, HbA1c, ln HOMA-IR, and IRI were included as explanatory variables in the method.

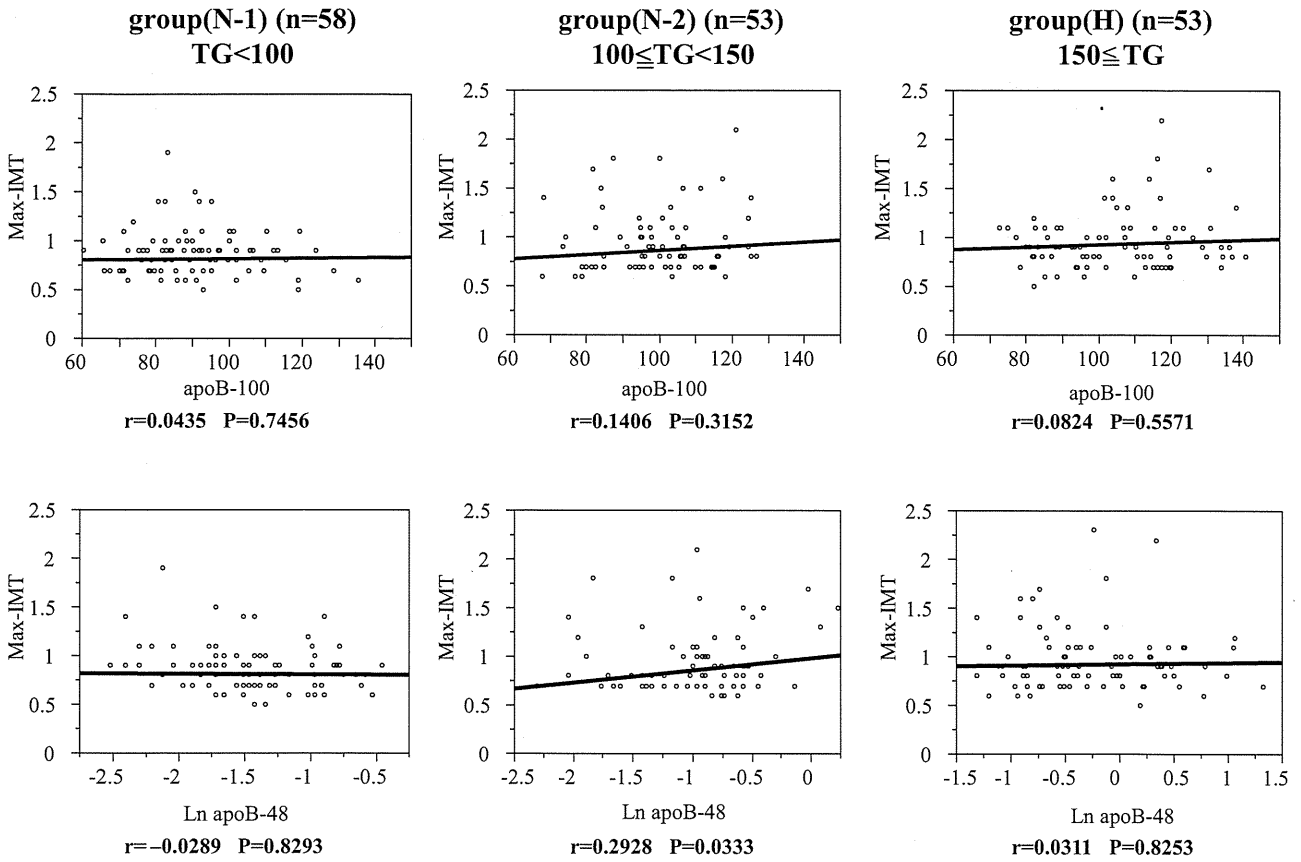



**Fig. 3.** Correlations between fasting apoB-100 levels or fasting Ln apoB-48 levels and max-IMT. There was no significant correlation between fasting apoB-100 levels and max-IMT in each TG group. Although the correlations between fasting apoB-48 levels and max-IMT in group N-1 and group H were not significant, there were a significant correlation between fasting apoB-48 levels and max-IMT in group N-2 as assessed by Pearson's correlation coefficients ($P < 0.05$).

features [11]. ApoB-48 was identified *in vivo* in human atherosclerotic plaques from femoral and carotid endarterectomy samples [21]. CM-R were shown to cause foam cell formation of mouse peritoneal and human monocyte-derived macrophages *in vitro* by both LDL-receptor-dependent and -independent mechanisms [11,22], stimulate MCP-1 expression in cultured vascular smooth muscle cells (VSMCs) [23], induce early growth response factor-1 (Egr-1) and proinflammatory cytokines, such as interleukin-2 (IL-2) and interferon-γ (IFN-γ) in VSMCs [24], increase the production of plasminogen activator inhibitor-1 (PAI-1) in endothelial cells via the MAPK pathway and redox system [25] and enhance endothelial cell apoptosis [26]. We found that fasting apoB-48 level was an independent risk factor for coronary stenosis assessed by coronary angiography (OR of apoB-48; 6.4, 95% CI; 3.64–1.79) (Masuda et al., unpublished observation). The increase in carotid IMT is significantly correlated with CHD and stroke [27]. Only a few studies have

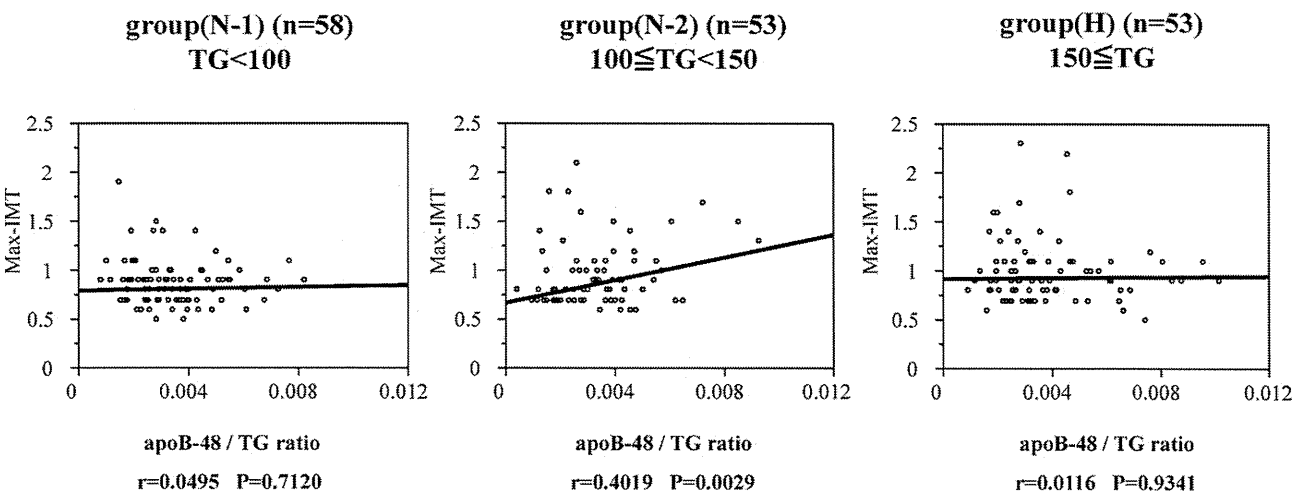



**Fig. 4.** Correlations between apoB-48/TG ratio and max-IMT.
The correlation between apoB-48/TG ratio and max-IMT was not significant in group N-1 and group H, but there was a significant correlation between apoB-48/TG ratio and max-IMT in group N-2 as assessed by Pearson's correlation coefficients ($P < 0.05$).

shown that there was a highly significant, independent correlation between the postprandial TG response and IMT [10], and that the presence of carotid plaque was associated with fasting apoB-48 and TG levels in age- and gender-adjusted analysis in type 2 diabetic patients [17]. As shown in the current study, the increase in apoB-48-containing lipoproteins, mainly CM-R, had a significant relationship with max-IMT. ApoB-48 level was also shown to be an independent variable of max-IMT in group N-2 (Fig. 3 and Table 2) which may significantly affect the development of systemic atherosclerosis associated with CHD and stroke. Ln apoB-48 level was associated with max-IMT, but LDL-C or apoB-100 levels were not correlated (Table 1 and Figs. 3 and 4). Tanimura et al. [17] also showed that the presence of carotid plaque was associated with high fasting apoB-48 levels but not with fasting TG levels in subjects with normal LDL-C (<140 mg/dl) levels. It was speculated that the impaired clearance and the accumulation of CM-R might be linked to carotid IMT and the development of atherosclerotic cardiovascular diseases, independent of the impaired clearance of VLDL and LDL.

### 4.2. CM-R particle size and atherogenic status

The size of CM produced by the small intestines is too large to penetrate the arterial wall; however, through the hydrolysis of TG by lipoprotein lipase (LPL) CM-R can become small enough to penetrate the arterial wall, be retained in the subendothelial space and affect the development of atherosclerotic plaques [11]. As shown in our former study, the size of CM-R changes from that of CM to that of HDL in the postprandial state [28]. Interestingly, in the current study, there was a strong correlation between apoB-48/TG ratio and max-IMT in group N-2 (Fig. 4). The high ratio of apoB-48/TG indicates that the number of apoB-48-containing lipoprotein particles increased while the number of TG components of these lipoproteins decreased, suggesting that the number of small-sized CM-R increased. The correlation between ln apoB-48 level and max-IMT in group N-2 whose TG levels were small indicates that the increase of small-sized CM-R was associated with the development of carotid atherosclerosis. Thus, serum apoB-48 level might be a good marker for the detection of early atherosclerosis in middle-aged, normotensive subjects with normal TG level.

### 4.3. Other metabolic phenotypes and apoB-48 levels

In subjects with high TG level (group H), there is a strong risk factor for the development of atherosclerosis. BMI, waist circumference, TG, apoB-48, RemL-C and IRI levels were significantly higher and HDL-C levels were significantly lower in group H than in group N-2 (Table 1), indicating that subjects in group H were capable of accumulating abdominal visceral fat which strongly affects insulin resistance or adipocytokine dysregulation. However, there was no significant correlation between ln apoB-48 level and max-IMT (Fig. 3). This discrepancy might be due to the clearance of CM and CM-R in subjects with abdominal visceral fat accumulation. The existence of insulin resistance deteriorates the lipoprotein metabolism of apoB-48-containing lipoproteins as well as apoB-100 -containing lipoproteins, which has been mainly explained by the impaired activity of LPL [29]. In these patients, low LPL activity causes an accumulation of large-sized CM-R or VLDL-R, resulting in an increase in TG and apoB-48. It was suggested that this buildup in large-sized lipoproteins was not precisely correlated with the enhancement of atherogenicity. There was no significant difference in LDL-C and apoB-100 levels among each TG group (Table 1), and fasting TG levels were mainly related to the accumulation of CM-R, less related to insulin resistance or apoB-100-containing lipoprotein metabolism. We could not find a positive correlation between RemL-C and max-IMT in our study subjects. The increase in CM-R did not properly reflect the increase in remnant lipoprotein cholesterol (RemL-C) levels which were shown to consist of CM-R and VLDL remnants. These CM-R and VLDL remnants have different origins, and their serum concentrations may vary depending upon the impairment of different pathways of lipoprotein metabolism. Above all, in the current study, there was a significantly positive correlation between apoB-48 level and max-IMT in group N-2, which was mainly associated with the increase in small-sized CM-R.

### 4.4. Limitation of the study

We aimed at evaluating the effectiveness of apoB-48 measurement in healthy subjects for the prediction of asymptomatic carotid atherosclerotic change. We recruited only men and focused on subjects coming to the Osaka Police Hospital, and therefore these factors are supposed to be the baseline of the bias.

## 5. Conclusion

In conclusion, these data suggest that the accumulation of CM-R might be an independent risk factor for the development of atherosclerosis among subjects with TG levels between 100 mg/dl and 150 mg/dl. The measurement of fasting apoB-48 level is very useful for the detection of early onset of atherosclerotic plaques.

## Funding sources

This work was supported by the following grants: a grant-in-aid for Scientific Research (No. 18659267) to Yamashita S from the Ministry of Education, Science, Sports and Culture in Japan; a grant from Mitsui Life Social Welfare Foundation to Yamashita S; a Takeda Medical Research Foundation Grant to Yamashita S.

## Disclosure

S. Yamashita is a member of Fujirebio's Advisory Boards (Tokyo, Japan). He has received compensation for travel expenses for Conference Lectures supported by this company. Co-authors do not wish to disclose any other matter.

## Acknowledgements

We gratefully acknowledge Fujirebio Inc. (Tokyo, Japan), Sekisui Medical Co., Ltd. (Tokyo, Japan) and Kyowa Medex Co. (Tokyo, Japan) for measuring our samples with high quality standards. We also acknowledge the excellent technical and administrative assistance extended by K. Hizu, R. Wada and M. Kato.

## References

[1] Kannel WB, Castelli WP, Gordon T, McNamara PM. Serum cholesterol, lipoproteins, and the risk of coronary heart disease. The Framingham study. Ann Intern Med 1971;74(1):1–12.
[2] LaRosa JC, Grundy SM, Waters DD, et al. Treating to New Targets (TNT) Investigators. Intensive lipid lowering with atorvastatin in patients with stable coronary disease. N Engl J Med 2005;352(14):1425–35.
[3] Albrink MJ, Man EB. Serum triglycerides in coronary artery disease. AMA Arch Intern Med 1959;103(1):4–8.
[4] Austin MA. Plasma triglyceride and coronary heart disease. Arterioscler Thromb 1991;11(1):2–14.
[5] Eberly LE, Stamler J, Neaton JD. Relation of triglyceride levels, fasting and nonfasting, to fatal and nonfatal coronary heart disease. Arch Intern Med 2003;163(9):1077–83.
[6] Iso H, Naito Y, Sato S, et al. Serum triglycerides and risk of coronary heart disease among Japanese men and women. Am J Epidemiol 2001;153(5):490–9.
[7] Castelli WP. Lipids, risk factors and ischamemic heart disease. Atherosclerosis 1996;124(Suppl.):S1–9.
[8] Mori Y, Itoh Y, Komiya H, et al. Association between postprandial remnant-like particle triglyceride (RLP-TG) levels and carotid intima-media thickness (IMT)