OX40 ligand plays an important role in the development of atherosclerosis through vasa vasorum neovascularization

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Aims
Atherosclerosis is characterized by infiltration of inflammatory cells and enhanced vasa vasorum formation, for which immunological mechanisms may be involved. OX40, a membrane-bound molecule of the tumour necrosis factor-receptor superfamily, is expressed by activated T-cells, while OX40 ligand (OX40L) is expressed in activated macrophages and endothelial cells. In this study, we thus examined whether the OX40/OX40L system is involved in the pathogenesis of atherosclerosis.

Methods and results
We examined apolipoprotein E-deficient (ApoE⁻/⁻) mice and ApoE⁻/⁻/OX40L-double-deficient (ApoE⁻/⁻/OX40L⁻/⁻) mice fed on a high-fat diet for 8 weeks. The extent of aortic atheroma was significantly less in ApoE⁻/⁻/OX40L⁻/⁻ mice compared with ApoE⁻/⁻ mice. We also treated high-fat-fed ApoE⁻/⁻ mice with or without MGP34 antibody (OX40L-specific neutralizing antibody) for 10 weeks. After the treatment, the extent of aortic atheroma was again significantly less in MGP34-treated mice compared with controls. Importantly, both vascular density in the aortic adventitia and vascular endothelial growth factor-induced angiogenesis in the Matrigel assay in vivo were significantly reduced in ApoE⁻/⁻/OX40L⁻/⁻ mice compared with ApoE⁻/⁻ mice. Finally, when high-fat-fed ApoE⁻/⁻ mice were transplanted with bone marrow cells from either wild-type or OX40L⁻/⁻ mice, the extent of aortic atheroma was comparable between the two groups.

Conclusion
These results indicate that the vascular OX40/OX40L system plays an important role in the formation of vasa vasorum and subsequent atherosclerosis, suggesting that the vascular OX40/OX40L system might be a new therapeutic target of atherosclerosis.

Keywords
Atherosclerosis • Angiogenesis • Immune system

1. Introduction
Atherosclerosis is the major cause of death in western countries, accounting for ~50% of all deaths through the development of acute coronary syndrome, stroke, and cardiac sudden death. Although lipid-lowering and anti-hypertensive drugs are widely used for anti-atherosclerosis therapy,¹ more effective therapies need to be developed for the treatment of the common disorder. Since atherosclerotic lesions contain abundant immune cells (e.g. macrophages and lymphocytes) that play key roles in the pathogenesis of atherosclerosis, such immune cells and related ligand/receptor system on the cells could be novel therapeutic targets.²–⁵ OX40 is a membrane-bound molecule of the tumour necrosis factor-receptor superfamily and is expressed in activated T-cells, while OX40 ligand (OX40L) is expressed in activated macrophages and endothelial cells.⁶ T-cells also facilitate post-ischaemic angiogenesis by recruiting macrophages to ischaemic tissues, promoting secretion of cytokines in murine hindlimb ischaemia models.⁷,⁸ Moreover, neovascularization from vasa vasorum microvessels, whose functions were originally thought to be the pathways of nutrients and oxygen to blood vessel wall, play important roles in the development of atherosclerotic plaques in both animals and humans.⁹–¹³ However, it remains to be...
examined whether the immune system contributes to the development of vasa vasaorum neovascularization with a resultant development of atherosclerosis.

We and others have previously demonstrated the important protective role of bone marrow (BM)-derived cells, where endothelial progenitor cells are mobilized to ischemic tissue and promote post-ischaemic angiogenesis, play pivotal role in the maintenance of endothelial function in flow-mediated vasodilation examination, and exert a protective effect on hypoxia-induced pulmonary arterial hypertension (PAH). In contrast, BM-derived vascular smooth muscle cells have been shown to be involved in the pathogenesis of vascular lesions, including PAH, injury-induced arteriosclerosis, and high-fat-induced atherosclerosis, suggesting the divergent roles of BM-derived cells. Furthermore, we and others have previously demonstrated that the inhibition of OX40 ligand significantly reduces atherosclerotic lesion in mice. Although previous study showed the importance of the OX40/OX40L system on atherogenesis through down-regulation of IL-4 and increment of IgM to oxidized low-density lipoprotein, the importance of OX40/OX40L on the development of atherosclerosis through both vasa vasaorum neovascularization and recruitment of BM-derived cells remains unclear.

In the present study, we thus examined whether the OX40/OX40L system is involved in the pathogenesis of atherosclerosis in apolipoprotein E-deficient (ApoE−/−) mice, with a special reference to vasa vasaorum neovascularization and the interaction between BM-derived cells and local vasculature.

2. Methods
All procedures were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University, which was granted by Tohoku University Ethics Review Board (No. 76-20-236) and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 85-23, revised 1996).

2.1 Animal preparation
In the genetic deletion protocol, OX40L-deficient (OX40L−/−) mice were bred with ApoE−/− mice (Jackson Laboratory, Bar Harbor, ME, USA). Genotyping for OX40L and ApoE was performed by polymerase chain reaction. Female ApoE−/− mice and ApoE−/−/OX40L−/− mice were maintained on a western-type diet (1.25% cholesterol, 16.5% fat, 20% carbohydrate) and high-fat-induced atherosclerosis, suggesting the divergent roles of OX40L in atherogenesis.

2.2 Blood and plasma analysis
A total of 0.5–1.0 mL of blood was obtained from mice by inferior vena cava puncture when they were euthanized. A small aliquot of blood was analysed for a complete blood count (Drew Scientific Group, Oxford, CT, USA), and the remainder was used for the enzymatic colorimetric analysis of total cholesterol and triglyceride levels (Wako Roche Diagnostics, Indianapolis, IN, USA).

2.3 Bone marrow transplantation
Female ApoE−/− mice were lethally irradiated and transplanted with 5 × 106 donor BM cells from either wild-type (WT) or OX40L−/− mice (n = 9 each), some of which were from GFP-positive WT or GFP-positive OX40L−/− mice (n = 4.5, respectively). The BM cells were obtained by flushing the tibias and femurs of donor mice, suspended in 100 μL calcium- and magnesium-free PBS with 2% foetal bovine serum. The chimeric rate was more than 95% by FACS analysis. The animals were maintained on a high-fat diet for 8 weeks after the transplantation, followed by the examination of the extent of aortic atheroma.

2.4 En face lesion analysis
To quantify the extent of aortic lesions, en face preparations of the aorta were determined by ORO staining. Briefly, the aorta was fixed with 10% formalin, opened longitudinally, and pinned on the surface of black wax with steel pins. After that, they were stained with ORO solution and washed in 85% propylene glycol solution. Images were obtained with a Nikon Coolpix camera (Nikon Inc., Tokyo, Japan) attached to an inverted microscope. The area of ORO stained lesion was determined by AxioVision (Carl Zeiss, Jena, Germany).

2.5 Aortic root lesion analysis and immunohistochemistry
The heart and the aortic root were dissected and fixed with 4% paraformaldehyde. Cryosections were stained with ORO solution and haematoxylin. Immunohistochemistry was performed with anti-mouse α-actin (1:400, DAKO, Glostrup, Denmark), Mac3 (1:100, BD Pharmingen), and CD31 (1:100, BD Pharmingen). Negative controls were prepared by substitution with an isotype control antibody. The area of ORO-stained lesion and immunopositive area was quantitatively analysed by AxioVision (Carl Zeiss, Jena, Germany) in cross-sections obtained at the level of all three leaflets of the aortic valve, immediately proximal to the right coronary artery ostium.

2.6 Matrigel assay
The Matrigel (Becton Dickinson) implantation assay was performed as previously described. Briefly, 200 μL of growth factor-reduced Matrigel containing vascular endothelial growth factor (VEGF) (Invitrogen, 100 ng/mL) plus heparin (20 IU/mL) was injected into the abdominal subcutaneous tissue of each mouse. The gels were removed on day 21 and the sections of the gels were stained with anti-mouse CD31 (1:100, BD Pharmingen). We have manually counted the number of microvessels in high power fields.

2.7 Statistical analysis
The results are expressed as means ± SD. Statistical analyses were performed with StatView (StatView 5.0, SAS Institute Inc., Cary, NC, USA). Group means were compared with Student’s t-test. Comparisons of parameters between the two groups under different conditions were made by two-way ANOVA, followed by Bonferroni’s post hoc test. A value of P 0.05 was considered to be statistically significant.

3. Results
3.1 Genetic deletion of OX40L suppresses atherosclerosis in ApoE−/− mice
To examine whether the inhibition of the OX40/OX40L system suppresses early atherosclerotic lesion in ApoE−/− mice, ApoE−/− mice and ApoE−/−/OX40L−/− mice were fed on a high-fat diet for 8 weeks, starting at 4 weeks of age. After 8 weeks, there was no significant difference in body weight, total cholesterol or triglyceride values,
or white blood cell count between the two genotypes (Table 1). First, we performed aortic en face analysis with ORO staining, which demonstrated that the extent of atherosclerosis was significantly less in ApoE\(^{-/-}\)/OX40L\(^{-/-}\) mice compared with ApoE\(^{-/-}\) mice (Figure 1A and B). Next, we performed aortic root analysis with ORO staining, which demonstrated that the extent of aortic atheroma also was significantly less in ApoE\(^{-/-}\)/OX40L\(^{-/-}\) mice compared with ApoE\(^{-/-}\) mice (Figure 1C and D). In contrast, accumulation of macrophages and contents of smooth muscle cells in atheroma was comparable between the two genotypes (Figure 1E–H).

### 3.2 Inhibition of OX40L by antibody treatment suppresses atherosclerosis in ApoE\(^{-/-}\) mice

Next, we examined whether the inhibition of OX40L by neutralizing antibody influences the developed atherosclerotic lesion in ApoE\(^{-/-}\) mice. ApoE\(^{-/-}\) mice at 10 weeks of age were maintained on a western-type diet, while treated with either MGP34 antibody (OX40L-specific neutralizing antibody) or a control non-immune antibody (rat IgG), for 10 weeks. After 10 weeks, there was no significant difference in body weight, total cholesterol or triglyceride values, or white blood cell count between the two groups (Table 1). The extent of aortic atheroma in en face tended to be reduced in the MGP34-treated group, although not statistically significant (Figure 2A and B). However, the extent of atheroma in the aortic root was significantly reduced in the MGP34-treated ApoE\(^{-/-}\) mice than in the control IgG-treated ApoE\(^{-/-}\) mice (Figure 2C and D). In contrast, accumulation of macrophage and contents of smooth muscle cells in atheroma were again comparable between the two groups (Figure 2E–H).

### 3.3 Inhibition of OX40L suppresses vasa vasorum neovascularization in ApoE\(^{-/-}\) mice

A recent study has shown that vasa vasorum neovascularization substantially contributes to the development of atherosclerosis. To elucidate the role of OX40L in vasa vasorum neovascularization for atherogenesis, we examined adventitial vascular density by immunostaining and confirmed that the number of blood vessels in the adventitia was significantly less in ApoE\(^{-/-}\)/OX40L\(^{-/-}\) mice compared with ApoE\(^{-/-}\) mice (Figure 3A and B). To further investigate the role of the OX40/OX40L system in vasa vasorum neovascularization, we subcutaneously injected Matrigel with or without VEGF (100 ng/mL) into the abdomen of WT and OX40L\(^{-/-}\) mice. On day 21 after the injection, we observed VEGF-induced angiogenesis in the WT mice, but not in the OX40L\(^{-/-}\) mice (Figure 3C and D).

### 3.4 No role of OX40L in bone marrow cells in aortic atheroma in ApoE\(^{-/-}\) mice

Finally, we examined whether the OX40/OX40L system originated from BM-derived cells contribute to the development of atherosclerosis. High-fat-fed ApoE\(^{-/-}\) mice were transplanted with BM cells from either WT or OX40L\(^{-/-}\) mice. After 8 weeks of transplantation, there was no significant difference in total cholesterol or triglyceride values between the two genotypes (Figure 4A and B). En face analysis shows that the extent of aortic atheroma and homing of GFP-positive cells also were comparable between the two genotypes (Figure 4C–F).

### 4. Discussion

The novel findings of the present study are that (i) inhibition of the OX40/OX40L system, either by genetic deletion or by a neutralizing antibody, suppresses both early and developed atherosclerotic lesions in ApoE\(^{-/-}\) mice, at least in part, through inhibition of vasa vasorum neovascularization and (ii) OX40L in blood vessels, but not that in BM cells, contributes to the pro-atherogenic effects of the OX40/OX40L system. To the best of our knowledge, this is the first study that demonstrates that the vascular OX40/OX40L system promotes the development of atherosclerosis, at least in part, through vasa vasorum neovascularization.

### 4.1 Inhibition of OX40L reduces angiogenesis in atherosclerotic vascular wall

Diffusion of oxygen from vascular lumen to the vascular wall is limited when the vascular wall thickness exceeds more than 100 \(\mu m\). Progression of atheroma enhances vascular wall thickening, resulting in reduced oxygen content in the vascular wall with a resultant accumulation of hypoxic inducible transcriptional factors (HIFs), such as HIF-1, that induce expression of various angiogenic cytokines. Accumulating macrophages in ischaemic tissue also secrete angiogenic factors, which further enhance angiogenesis.
cytokines that facilitate the development of collateral blood vessels.\textsuperscript{7} Angiogenesis in the arterial wall is also stimulated by hypoxia-independent pathways, mediated primarily by inflammation and activation of the toll-like receptors.\textsuperscript{27} Furthermore, it was shown that microvessels are co-localized with macrophages and T lymphocytes in human vulnerable atheroma.\textsuperscript{28} In the present study, we were able to demonstrate that the genetic deletion of the OX40/OX40L signalling suppresses the development of vasa vasorum neovascularization in arterial wall and inhibits the development of atherosclerosis. It is conceivable that angiogenic cytokines from atheroma promote angiogenesis in the arterial wall, recruiting immune cells that cause continuous secretion of cytokines and further recruitment of immune cells.\textsuperscript{29} Newly formed blood vessels are leaky to allow the extravasation of red blood cells, again promoting immune cell infiltration into the arterial wall.\textsuperscript{26,30,31} Various inflammatory cytokines could activate matrix-metalloproteinases, which also promote angiogenesis as well as plaque disruption.\textsuperscript{32–34} Thus, it is possible that once angiogenesis and recruitment of immune cells begin, the vicious cycle of inflammation would further enhance the development of atherosclerosis. In the present study, we were also able to demonstrate that inhibition

Figure 1 Atherosclerotic lesion in ApoE\textsuperscript{−/−} mice and ApoE\textsuperscript{−/−}/OX40L\textsuperscript{−/−} mice that were fed a high-fat diet for 8 weeks. (A) Representative ORO staining of en face aorta. (B) Quantification of ORO staining of en face aorta. (C) Representative ORO staining of aortic root. (D) Quantification of ORO staining of aortic root. (E) Representative immunohistochemistry of Mac-3 staining of aortic root. (F) Quantification of Mac-3 staining of aortic root. (G) Representative immunohistochemistry of α-actin staining of aortic root. (H) Quantification of α-actin staining of aortic root. Results are expressed as means ± SD.
of the OX40/OX40L system reduces VEGF-induced angiogenesis in vivo. The OX40/OX40L system is known to activate T cells, which can produce cytokines and result in priming macrophages activation. It has been demonstrated that mononuclear phagocyte lineage cells, such as macrophages and dendritic cells, which are known to be major cellular components of innate immunity, play important roles in the development of atherosclerotic lesion. T cells, which are one of the key components of adaptive immunity, are also known to enhance inflammation in vascular wall and promote atherogenesis. A previous study has indicated that genetic deletion of CD4 T cells inhibits VEGF-secretion from macrophages in ischaemic tissue. Taken together, it would be possible that the OX40/OX40L system develops atherosclerosis partly by facilitating vasa vasorum neovascularization through both innate and adaptive immunity. Moreover, it has recently reported that the OX40/OX40L system activates phospholipase C, which induces diacylglycerol-protein kinase C (DAG-PKC) and the inositol triphosphate (IP3)-intracellular free calcium ([Ca2+]i) pathway in HUVEC. These pathways are also known as down-stream signal pathway of VEGF-induced angiogenesis. Therefore, it would be possible that the OX40/OX40L system

Figure 2 Atherosclerotic lesion in ApoE<sup>−/−</sup> mice that were fed a high-fat diet and treated with either control IgG or MGP34 antibody for 10 weeks. (A) Representative ORO staining of en face aorta. (B) Quantification of ORO staining of en face aorta. (C) Representative ORO staining of aortic root. (D) Quantification of ORO staining of aortic root. (E) Representative immunohistochemistry of Mac-3 staining of aortic root. (F) Quantification of Mac-3 staining of aortic root. (G) Representative immunohistochemistry of α-actin staining of aortic root. (H) Quantification of α-actin staining of aortic root. Results are expressed as means ± SD.
facilitates angiogenesis through enhancing VEGF induction in inflammatory cells and directly activating DAG-PKC and IP3-[Ca2+]) pathways. Taken together, the present results indicate that the inhibition of the OX40/OX40L system suppresses hypoxia-induced inflammatory responses in the atherosclerotic arterial wall. A previous study has shown that interruption of the OX40/OX40L system diminished atherosclerosis in LDL⁻/⁻/² mice through down-regulation of IL-4, which is known as one of the proangiogenic cytokines in murine hypoxic lung. Thus, it would be possible that the inhibition of IL-4 partly contributes to suppression of angiogenesis in atherosclerotic lesion.

4.2 Important role of vascular OX40L in atherogenesis

Since atherosclerotic lesions have many inflammatory cells derived from BM, many attempts have been made to suppress molecules on BM cells to stabilize atherosclerotic plaques without success. In the present study, we have indicated the insignificant differences in the extents of atheroma and homing of BM cells between ApoE⁻/⁻ with WT-BM and ApoE⁻/⁻ with OX40L⁻/⁻-BM, suggesting that OX40L in blood vessels, but not that in BM cells, substantially contributes to atherogenesis in ApoE⁻/⁻ mice. A similar result has been obtained in a recent study, showing that MMP-9 in blood vessels rather than that in BM cells is required for plaque development. Taken together, the present results suggest that vascular OX40L, but not that in BM, plays an important role in the pathogenesis of atherosclerosis.

4.3 Limitation of the study

Several limitations should be mentioned for the present study. First, the treatment with the MGP34 antibody did not completely mimic that of genetic deletion of OX40L. However, it is widely known that neutralizing antibody does not have complete inhibitory effect on the target molecule when compared with genetic deletion. Second, we could not detect the difference of atheromatous components (e.g. macrophages or smooth muscle cells), although the
previous studies have also shown the insignificant differences in these contents in spite of different volume of lipid deposition. It might be possible that such results depend on models and target molecules in cases. Fourth, detailed molecular mechanisms of the OX40L expression remain to be examined in future studies.

4.4 Clinical implications

Previous studies suggested that suppression of immune molecules could reduce atherosclerosis; however, such strategies could impair host defenses because of systemic immunosuppressive effects. As shown in recent study, inhibition of the OX40/OX40L system is not associated with systemic immunosuppression. Thus, the OX40/OX40L system could be regarded as an effective therapeutic target for the treatment of atherosclerosis without adverse effects, although further studies are required.

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