Vascular endothelial cell-derived endothelin-1 mediates vascular inflammation and neointima formation following blood flow cessation

Dyah W. Anggrahini1, Noriaki Emoto1,3*, Kazuhiko Nakayama1, Bambang Widyantoro1, Suko Adiarto1, Naoko Iwasa1, Hitomi Nonaka1, Yoshiyuki Rikitake2, Yaz Y. Kisanuki4, Masashi Yanagisawa5, and Ken-ichi Hirata1

1Division of Cardiovascular Medicine, Department of Internal Medicine, 7-5-1 Kusunoki, Chuo, Kobe 650-0017, Japan; 2Division of Molecular and Cellular Biology, Department of Biochemistry and Molecular Biology, Kobe University Graduate School of Medicine, Kobe, Japan; 3Clinical Pharmacy, Kobe Pharmaceutical University, Kobe, Japan; 4Department of Neurology, The University of Michigan, Ann Arbor, MI, USA; and 5Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, USA

Received 5 November 2008; revised 15 January 2009; accepted 18 January 2009; online publish-ahead-of-print 22 January 2009

Time for primary review: 22 days

Aims Although endothelin-1 (ET-1) has been suggested to contribute to the pathogenesis of neointima formation and atherosclerosis, the individual roles of ET-1 derived from certain cell types in this disease remain unclear. In this study, we determined the role of vascular endothelial ET-1 on vascular inflammation and neointima formation using vascular endothelial ET-1-knockout [ET-1f/f; Tie2-Cre (+)] mice.

Methods and results Intimal hyperplasia was induced by complete ligation of the left carotid artery in 12-week-old male ET-1f/f;Tie2-Cre (+) mice (n = 35) and the wild-type (WT) littermates (n = 34). Following this intervention, neointima formation was reduced in ET-1f/f;Tie2-Cre (+) mice compared with the WT mice, independent of the difference in blood pressure. This reduction was associated with a decrease in inflammatory cell recruitment to the vessel wall, which was accompanied by reduced expression levels of endothelial adhesion molecules as well as chemokines and a decrease in vascular smooth muscle cell proliferation.

Conclusion The results of our study provide direct evidence for the role of vascular endothelial ET-1 in mediating vascular inflammation and neointima formation following vascular injury in addition to promoting vasoconstriction and cell proliferation. Furthermore, this study suggests a strategy for the efficient design of ET receptor antagonists with targeted inhibition of ET-1 signalling in vascular endothelial cells.

KEYWORDS
Endothelin-1; Atherosclerosis; Neointima formation; Inflammation; Carotid ligation

1. Introduction

Inflammation of the vascular wall and vascular smooth muscle cell (VSMC) proliferation in response to haemodynamic changes in the vessel wall are primary mediators in the development of neointima lesion and vascular remodelling.1 Endothelial cells (ECs) play an important role in maintaining the vessel homeostasis in response to altered flow.2 Activation of these cells after changes in shear stress promotes the expression of adhesion molecules and the recruitment of inflammatory cells, followed by proliferation and migration of VSMCs, resulting in vascular remodelling.3–5

Endothelin-1 (ET-1), a 21-amino acid peptide, is known as the most potent vasoconstrictor and is secreted mainly in ECs.6 This peptide acts through two different G protein-coupled receptors, ETA and ETB.7,8 VSMCs express ETA and ETB receptors, which mediate vasoconstriction, whereas ETB receptors on vascular ECs mediate vasodilatation through the release of nitric oxide (NO) and prostacyclin.8,9 Moreover, in vitro studies suggested that ET-1 stimulates VSMC proliferation7,10 and mediates the interactions between leucocytes and ECs11 partially through its ability to stimulate the expression of adhesion molecules.12–14 Given the opposing effects of ET-1 binding to distinct receptors, and its function as a proliferating agent as well as a pro-inflammatory cytokine, ET-1 is thought to play an important role in a variety of vascular diseases.7–9
Over the last decade, a considerable effort has been made to better define the role of ET-1 in the intimal hyperplasia in atherosclerosis as well as in vascular remodelling. Increased ET-1 expression is found in patients with intimal hyperplasia and atherosclerosis. Studies using ET receptor antagonist have shown the significance of systemic inhibition of ET-1 action in the pathogenesis of neointima formation. Given the high expression of ET-1 in ECs, inflammatory cells, and VSMCs in the intimal hyperplasia, these studies using receptor blockade did not allow us to discriminate between the individual contributions of ET-1 derived from several types of cells in mediating the inflammation and neointima formation. Thus, it remains unclear by what type of cells ET-1 produced specifically play the most important role in the pathology of neointima formation in vascular remodelling.

Homozgyous deletion of ET-1 in mice results in craniofacial and cardiac abnormalities, and death from respiratory failure soon after birth. Thus, we have created mice with deletion of preproET-1 gene in ECs using Cre-loxP recombinase system. The mice develop normally and are fertile with an undetectable serum level of ET-1 (Y.Y.K. and M.Y., unpublished data). The use of these mice allowed us to distinguish the function of ET-1 secreted by ECs from those by other cells for mediating vascular inflammation and neointima formation. We utilized the established model of carotid artery ligation in these mice and showed that neointima formation after cessation of blood flow was reduced with the reduction of early inflammation in the vascular wall.

2. Methods

Further detailed materials and methods are given as supplementary information.

2.1 Preparation of ET-1f/f;Tie2-Cre (+) mice

The detailed description about the generation of ET-1f/f;Tie2-Cre (+) mice will be published elsewhere (Y.Y.K. and M.Y., unpublished data). Briefly, a mouse strain with ET-1 exon 2, which codes the entire mature ET-1 peptide, is flanked by two loxP sites. For vascular endothelium-specific targeting, we used Tie2-Cre transgenic mice that express Cre recombinase in a pan-endothelial fashion. Breeding those mice resulted in genetically modified mice harbouring deletion of the preproET-1 gene specifically in ECs.

For experimental purpose, 10–12-weeks-age heterozygous ET-1f/f; Tie2-Cre (+) mice and control ET-1f/f; Tie2-Cre (−) littersmates (wild-type–WT mice) were used. All animal experimental protocols were conducted according to the Guidelines for Animal Experiments at Kobe University Graduate School of Medicine.

2.2 Blood pressure measurement and hydralazine treatment

Blood pressure was measured using tail cuff methods (Muromachi Kikai, Japan). Hydralazine was added to drinking water at 250 mg/L starting at 5 weeks of age to lower blood pressure to a comparable level between both groups.

2.3 Mouse carotid artery ligation model

To induce vascular inflammation and neointima lesion formation, we performed ligation of the left common carotid artery as described previously. Prior to operation, mice were anaesthetized using sodium pentobarbital (0.05 mg/g body weight) injected intraperitoneally.

2.4 Morphological and morphometrical analyses

Haematoxylin–eosin staining was used for morphological analysis of neointima lesion formation and the vessel from sham-operated control. A total of 15 sections were measured for the morphometrical analysis. Digital images of the vessels were analysed using the NIH Image Software version 1.37 (http://rsb.info.nih.gov/ij/).

2.5 Immunostaining

The tissue expression of ET-1, endothelial adhesion molecules, and inflammatory cells was observed using immunostaining method with appropriate antibody.

2.6 Proliferation assay

For proliferation assay, sections were stained with mouse anti-proliferating cell nuclear antigen (PCNA) (1:1, Dako) and Dako Envision HRP-anti mouse (1:1, Dako) as secondary antibody. A total of five sections per each animal were used for the staining, and mean number of proliferating cells per animal was taken for statistical analysis.

2.7 Real-time polymerase chain reaction

Twenty nanograms of total RNA from carotid artery was extracted for the analysis of endothelin system and adhesion molecules mRNA expression levels. Quantitative real-time polymerase chain reaction (PCR) was performed in Superscript III Platinum One-Step Quantitative RT-PCR System (Invitrogen) using Applied Biosystem 7500 Real Time PCR System. Lux™ fluorogenic primer sequences were designed using D-LUX™ Designer Software (Invitrogen).

2.8 In vivo leucocyte and macrophage peritoneal recruitment

To study the accumulation of leucocytes and macrophages in our mice, thioglycollate-induced peritonitis model was used as described previously.

2.9 Statistical analysis

Results are presented as mean ± standard error of the mean. Means were compared using paired or unpaired Student’s t-test for analysis on blood pressures and heart rate, for morphometrical data and cell numbers. One-way ANOVA followed by Fisher’s PLSD test with equal or unequal variances was demonstrated for the data of preproET-1 and adhesion molecules mRNA expressions. Mean differences with P-value <0.05 were considered statistically significant.

3. Results

3.1 Haemodynamic profile and morphological changes in ET-1f/f;Tie2-Cre (+) mice

The ET-1f/f; Tie2-Cre (+) mice were born with no defects and grew healthy into adulthood. The detailed phenotype of these mice will be described elsewhere (Y.Y.K. and M.Y., unpublished data). Basal systolic blood pressure (SBP) was slightly but significantly lower in ET-1f/f; Tie2-Cre (+) mice (104.00 ± 2.06 mmHg) compared with WT mice (113.22 ± 2.49 mmHg). At 4 weeks after carotid ligation, no increase in SBP was found after cessation of flow, and the SBP remained significantly lower in ET-1f/f; Tie2-Cre (+) mice (P < 0.05 compared with that of the WT mice) (Table 1).
Four weeks after operation, no significant differences in luminal, medial, and total vessel areas were observed in the unligated left carotid arteries of the WT and ET-1f/f; Tie2-Cre (−) mice (sham operation) (Figure 1A). In contrast, flow cessation resulted in a substantial increase in the medial as well as in the intimal area in both genotypes, but the significantly smaller neointima formation was found in ET-1f/f;Tie2-Cre (+) mice (P < 0.05 compared with the WT mice) (Figure 1B). αSMA staining revealed that most of the cells in the neointima were VSMCs (Supplementary Figure). This was associated with a significant decrease in the intima-to-media (i/m) ratio [0.25 ± 0.07 vs. 0.83 ± 0.25; ET-1f/f;Tie2-Cre (+) mice vs. the WT mice] and an increase in the lumen area (P < 0.05 as compared with the WT mice). The medial area and total vessel wall as defined by the external elastic lamina and internal elastic lamina were not different between two genotypes (Figure 1B), suggesting similar degrees of vascular constriction in both genotypes in response to carotid ligation.

To determine whether differences in blood pressure influence neointima formation, hydralazine was administered to mice in drinking water. Hydralazine treatment reduced SBP of the WT mice by 7.67 ± 0.70 mmHg to the similar levels found in ET-1f/f;Tie2-Cre (+) mice (Table 1). This reduction did not influence the intimal thickening (6.30 ± 2.55) and i/m ratio, which remained significantly higher compared with those of ET-1f/f;Tie2-Cre (+) mice (Figure 1B). These findings suggest that vascular ECs-specific ET-1 contributes to the neointima formation following vascular injury. Thus, further investigations were focused on determining the local role of ET-1 in the neointima formation.

### 3.2 Expression of endothelin-1 after carotid ligation

Basal carotid artery preproET-1 mRNA levels were lower by three-fold in ET-1f/f;Tie2-Cre (+) mice compared with WT mice. Figure 1C further demonstrates that cessation of blood flow in the carotid artery significantly increased the preproET-1 mRNA levels only in WT mice after 1 day, 3 days, and 4 weeks of ligation. Significant differences in the preproET-1 mRNA expression levels between both genotypes were found 1 day, 3 days, and 4 weeks after the intervention (Figure 1C).

Using a specific monoclonal anti-human ET-1 antibody, we demonstrated a lack of staining in ECs of the carotid artery in the unligated mice (Figure 1D). Four week carotid ligation significantly increased the staining in ECs and VSMCs in the neointima area of WT mice but not ET-1f/f;Tie2-Cre (+) mice. There was no compensatory increase in the ET-1 expression in VSMCs in the medial area as well as in inflammatory cells in the adventitia of the ligated and unligated ET-1f/f;Tie2-Cre (+) mice (Figure 1D). These findings indicate that changes in ET-1 levels in ECs of the carotid artery correlated with the reduced neointima formation.

### 3.3 Leucocyte and macrophage recruitment to the vessel wall

Inflammatory cells were adhered on the endothelial surface at 3 and 7 days after carotid artery ligation. The number of adherent CD45-positive cells on the luminal surface was significantly reduced in ET-1f/f;Tie2-Cre (+) mice compared with WT mice (54.60 ± 3.94 vs. 20.75 ± 4.93 cells/lesion at 3 days and 6.3 ± 0.72 vs. 2.00 ± 1.00 cells/lesion at 7 days, respectively, P < 0.05) (Figure 2A and B). Using F4/80 to observe macrophage recruitment, we also demonstrated the reduction in macrophage accumulation in the ligated vessel of ET-1f/f;Tie2-Cre (+) mice compared with WT mice (Figure 2A). Inflammatory cells were also found in the adventitia of the ligated vessel 3 and 7 days after ligation, which might be in part related to the ligation procedure (data not shown).

To further assess the leucocyte and macrophage homing response and recruitment in the WT and ET-1f/f;Tie2-Cre (+) mice in vivo, we employed thioglycollate-induced peritonitis in these mice. This intervention resulted in the accumulation of neutrophils and macrophage in the peritoneal cavity of both genotypes. As expected, neutrophil accumulation was reduced in the peritoneal cavity of ET-1f/f;Tie2-Cre (+) mice compared with WT mice. Similarly, macrophage recruitment to the peritoneum of ET-1f/f;Tie2-Cre (+) mice was also reduced compared with WT mice (Figure 2C). These findings suggest that vascular endothelial ET-1 mediates the recruitment of inflammatory cells to the vessel wall after injury and that the accumulation of those cells is impaired in ET-1f/f;Tie2-Cre (+) mice.

### 3.4 Expression of endothelial adhesion molecules

To determine the mechanism by which vascular endothelial ET-1 mediates the recruitment of inflammatory cells to the

| Table 1 Blood pressure and heart rate before and after hydralazine administration |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Pre-ligation                    | SBP, mmHg       | MBP, mmHg       | DBP, mmHg       | HR, per min     |
| ET-1f/f;Cre (−)                 | 113 ± 2         | 86 ± 3          | 60 ± 2          | 705 ± 23        |
| ET-1f/f;Cre (+)                 | 104 ± 2         | 84 ± 2          | 64 ± 3          | 696 ± 21        |
| ET-1f/f;Cre (−) + hydralazine  | 111 ± 2            | 79 ± 3          | 64 ± 4          | 736 ± 20        |
| Post-ligation                  | 116 ± 2         | 89 ± 3          | 62 ± 4          | 721 ± 19        |
| ET-1f/f;Cre (+)                 | 101 ± 3         | 81 ± 2          | 63 ± 5          | 713 ± 26        |
| ET-1f/f;Cre (−) + hydralazine  | 103 ± 3          | 80 ± 5          | 59 ± 5          | 730 ± 17        |

All results are presented as mean ± SEM. Values were obtained from mean of five measurements of individual mice. ET-1, endothelin-1; SBP, systolic blood pressure; MBP, mean arterial blood pressure; DBP, diastolic blood pressure; HR, heart rate.

*P < 0.05 vs. ET-1f/f Cre (−) pre-ligation group.

**P < 0.05 vs. ET-1f/f Cre (−) post-ligation group.
vessel wall, we evaluated the expression levels of endothelial adhesion molecules and chemokines such as PECAM-1, ICAM-1, VCAM-1, and MCP-1 following carotid ligation in WT and ET-1f/f;Tie2-Cre (+) mice.

Immunohistochemical staining demonstrated that the expression of PECAM-1, ICAM-1, and VCAM-1 was markedly different between ET-1f/f;Tie2-Cre (+) and WT mice 3 days (Figure 3A) and 7 days (data not shown) after ligation. Similarly, the MCP-1 immunofluorescence was also less in ET-1f/f;Tie2-Cre (+) mice compared with WT mice 1 day after ligation. Further quantitative analysis using real-time PCR showed the distinct expression pattern for each gene (Figure 3B). The mRNA expression levels of PECAM-1 increased in WT mice and significantly decreased in ET-1f/f;Tie2-Cre (+) mice 1 day and 3 days after flow cessation. Ligation of the carotid artery induced ICAM-1 mRNA expression, and significant differences between ET-1f/f;Tie2-Cre (+) and WT mice were found at 3 days and 1 week after ligation. mRNA expression levels of VCAM-1 and MCP-1 were significantly reduced in ET-1f/f;Tie2-Cre (+) mice compared with WT mice only on the first day after ligation. These data demonstrate that vascular endothelial ET-1 mediates endothelial expression of these adhesion molecules and chemokines following vascular injury, and that the decreased recruitment of inflammatory cells to the vessel wall observed in this study may be in part due to the reduction in the expression of adhesion molecules in ET-1f/f;Tie2-Cre (+) mice.

3.5 Decreased cellular proliferation in the vasculature of ET-1f/f;Tie2-Cre (+) mice

Endothelin-1 is also known as a strong proliferation-promoting agent.7,10 To determine the local vascular effect of vascular endothelial ET-1 on mediating neointima formation, we examined proliferating cells in the vascular wall by PCNA staining. Proliferating cell nuclear antigen staining was not observed prior to 7 days following vascular injury. The number of the proliferating cells in the intima as well as in the media reached maximum at 7 days and decreased at 14 days after carotid ligation. At both times, the total number of PCNA-positive cells was markedly decreased in ET-1f/f;Tie2-Cre (+) mice compared with WT mice (9.8 ± 2.26 vs. 24.78 ± 2.94 cells/lesion and 5.2 ± 1.45 vs. 18.82 ± 3.42 cells/lesion, respectively, P < 0.05) (Figure 4A and B). These results suggest that vascular endothelial ET-1 mediates the cellular proliferation that further contributes to neointima formation.

3.6 Expression of endothelin receptors following carotid ligation

To analyse the mechanism by which vascular endothelial ET-1 mediates neointima formation following vascular injury, we assessed the mRNA expression levels of ETA and ETB receptor systems. The mRNA expression levels of both receptors were significantly lower in ET-1f/f;Tie2-Cre (+) mice compared with WT mice 7 days following vascular injury (P < 0.05). Lower tendency to WT mice was found...
in ET-1^{f/f};Tie2-Cre (+) mice at 4 weeks after ligation (Figure 4C).

4. Discussion

In this study, we observe the role of ECs-derived ET-1 in a complete ligation-induced vascular remodelling in a mouse which is a model of vascular failure to physiological adaptation. The disturbed shear stress conditions after flow cessation cause the vessel to undergo inward remodelling and shrink in luminal area because of the reduction in vessel diameter and dramatic neointima formation. Using this model, we showed that vascular inflammation and neointima formation after flow cessation-induced vascular injury were significantly reduced in ET-1^{f/f};Tie2-Cre (+) mice compared with WT mice. These findings suggest an important role of ET-1 specifically derived from ECs in mediating neointima formation. It should be noted that the role in lesion formation is independent of the changes in blood pressure. Although ET-1^{f/f};Tie2-Cre (+) mice showed slightly lower SBP, this difference was similar after carotid ligation. Moreover, the intimal thickening as well as i/m ratio remained significantly lower in ET-1^{f/f};Tie2-Cre (+) mice after blood pressure reduction in WT mice.

Several mechanisms contribute to the observed changes in ET-1^{f/f};Tie2-Cre (+)-ligated vessels, including the inhibition of endothelial adhesion molecule expressions, the reduced recruitment of inflammatory cells into the vessel wall, and the decrease in VSMCs proliferation. In this study, the expression level of the preproET-1 mRNA and protein was markedly increased following the disruption of blood flow. It has been reported in vitro that the ET-1 gene is regulated by shear stress. Moreover, the cessation of flow will cause inward remodelling directed by vascular tone. Our result further revealed that the prevention of the increase in the ET-1 level in ET-1^{f/f};Tie2-Cre (+) mice after ligation resulted from the inhibition of ET-1 secretion by ECs. Thus, since the expression level of ET-1 in other cells such as VSMCs, macrophages, leucocytes, and adipocytes in the adventitia was similar, the current results suggest that the reduction in vascular inflammatory responses and neointima formation corresponds to the inhibition of ET-1 in ECs. However, we could not exclude the possibility that small percentage of ET-1 was also blocked from other Tie-2-positive cells. In addition, we observed the dynamic pattern of ET-1 increase in WT mice at 1 day and 4 weeks after ligation and the decrease after 2 weeks ligation. Nevertheless, we still could not explain this phenomenon, and future study is needed to further clarify the pattern.
and mechanism of ET-1 expression changes in ECs after disturbed shear stress.

The results of the current study are consistent with the previous findings that show a contribution of ET-1 in the development of intimal hyperplasia in vascular remodelling in the atherosclerosis model,15 hypertension model,15,18 or vascular injury model.16 However, those studies using ET receptor blockade or vascular injury models with injured endothelium might not be sufficient to elucidate the role of ECs-derived ET-1. Thus, we have chosen the model in which ECs remain intact following carotid ligation in order to determine the direct role of ET-1 derived from ECs on vascular remodelling using genetic deletion of ET-1 specifically in ECs.

Figure 3 The expression of endothelial adhesion molecules and chemokines. (A) Representative histological sections from the ligated carotid arteries of ET-1f/f;Tie2-Cre (−) and ET-1f/f;Tie2-Cre (+) mice stained for PECAM-1, ICAM-1, VCAM-1, and MCP-1. Original magnification ×400 and ×630 for MCP-1. (B) mRNA level of PECAM-1, ICAM-1, VCAM-1, and MCP-1 of ET-1f/f;Tie2-Cre (−) and ET-1f/f;Tie2-Cre (+) groups. For real-time polymerase chain reaction, 20 ng/μL of total RNA extracted from the ligated carotid arteries were used. Bars represent mean ± SEM (n = 4, each group of time courses). #P < 0.05 vs. ET-1f/f;Tie2-Cre (−) at the same time course.
The adhesion and recruitment of inflammatory cells to the vessel wall are the primary contributors to the inflammatory response following vascular injury. The reduction of inflammatory cell recruitment to the vessel wall and to the inflamed peritoneum in ET-1f/f;Tie2-Cre (+) mice is consistent with the previous findings showing that ET-1 mediates leucocyte–EC interaction in DOCA-salt rats and stimulates neutrophil adhesion to ECs in vitro. The mechanism underlying decreased leucocyte recruitment to the vessels of ET-1f/f;Tie2-Cre (+) mice could be explained in part by the expression of adhesion molecules and cytokines by activated vascular ECs. In this study, we show the upregulation of VCAM-1 in ET-1f/f;Tie2-Cre (+) mice could be explained in part by the expression of adhesion molecules and cytokines by activated vascular ECs. This upregulation of VCAM-1 and MCP-1 in the WT mice 1 day after ligation and that the expression of these molecules was significantly decreased in ET-1f/f;Tie2-Cre (+) mice. It was reported that the prominence of VCAM-1 is restricted to early lesions and to regions of vasculature with disturbed flow. In vitro study also suggests that ET-1 enhances VCAM-1 expression in TNF-α-stimulated vascular ECs. Furthermore, we also demonstrated reduced expression of ICAM-1 and PECAM-1 in ET-1f/f;Tie2-Cre (+) mice at 3 and 7 days following vascular injury. Endothelin-1-stimulated aortic ECs, human coronary ECs, and rat cardiomyocytes are reported to induce the expression of ICAM-1 mediated by nuclear transcription factor-kappaB, which could be blunted by ET_A or ET_B antagonist. Nevertheless, the role of PECAM-1 in atherosclerosis has yet to be studied in detail, and no study has reported to the effect of ET-1 on PECAM-1. Thus, the reduced expression of adhesion molecules and chemokine in ET-1f/f;Tie2-Cre (+) mice after carotid ligation is in part due to the inhibition of ET-1 production from ECs, although other mechanism(s) might be involved.

Figure 4  Cellular proliferation and the expression level of ET_A and ET_B receptors. (A) Representative photomicrographs of the carotid arteries of ET-1f/f;Tie2-Cre (-) and ET-1f/f;Tie2-Cre (+) mice stained for proliferating cell nuclear antigen (PCNA) at 7 days after ligation. Arrowheads indicate proliferating cell nuclear antigen-positive cells. Original magnification x400. (B) Quantitative analysis of total proliferating cell nuclear antigen-positive cell numbers at 7 and 14 days after ligation (n = 5 each group). (C) ET_A and ET_B receptor mRNA expression at 1 and 4 weeks after ligation in ET-1f/f;Tie2-Cre (-) and ET-1f/f;Tie2-Cre (+) mice. Twenty nanograms of total RNA was used for real-time polymerase chain reaction analysis. Bars represent mean ± SEM (n = 3-4 each group). #P < 0.05 vs. ET-1f/f;Tie2-Cre (-) at the same time course.
secretion, which further inhibits cells proliferation. The cooperative effect of ET-1 in mitogenesis with platelet-derived growth factor (PDGF), fibroblast growth factor-2, and epidermal growth factor in human coronary SMCs' proliferation, which is mediated by ETA receptor, was demonstrated in vitro.10,36 Further confirmation is needed to elucidate whether the reduced cell proliferation in this model is due to the autocrine effect of ET-1 or to the decrease in leucocyte recruitment, which further inhibits inflammatory and growth factor stimuli. Recent study also challenged the idea that circulating progenitor cells may home in to the site of vascular injury and transdifferentiate into VSMCs, contributing to neointima formation.37,38 In this regard, we cannot exclude the possibility that ET-1 contributes to this process. No report has documented the role of ECs-derived ET-1 or endothelial progenitor cells differentiation. Studies to elucidate those roles and the mechanisms await further investigation. In this study, we also did not evaluate the migration of VSMCs from media to intima, which could also lead to neointima formation. It was shown that ET-1 induced human coronary VSMCs migration in combination with PDGF-BB and angiotensin II in vitro.39,40 Hence, such a mechanism cannot be excluded.

We demonstrate here that in ligated vessels of ET-1+/−, Tie2-Cre (+) mice, both ETA and ETB mRNA expressions were significantly reduced at 1 week and showed a tendency to be reduced after 4 week ligation. ETA receptor signalling mediates proliferation of VSMCs,10,36 and the blockade of ETA-selective antagonist reduced inflammatory cells adhesion through the inhibition of adhesion molecules,31,32 The ETB receptor is expressed in ECs and mediates ET-1-induced leucocyte–endothelial interaction through the activation of VCAM-1 expression.11,12 Although we did not perform any receptor-binding activity assays, our results on mRNA expression levels of both receptors could explain the mechanism by which ECs-derived ET-1 mediates early inflammation and neointima formation following vascular injury.

The possible mechanism that NO is involved in this model cannot be excluded. Flow alterations induce the activation of endothelium including the release of ET-1 and NO.41 The increased production of ET-1 in the WT animal in our study might reduce the eNOS-derived NO to inhibit the expression of adhesion molecules and inflammation, which further cause lesion formation.12,42 The limitation of the present study is that our current result is within the scope of the present model rather than the generalized interpretation for human atherosclerosis since the pathophysiology is significantly different and the precise mechanism of neointima formation in this model is still unclear.

In summary, the present study showed for the first time the role of EC-derived ET-1 in mediating inflammatory cell recruitment and neointima formation induced by ligation of the carotid artery. Endothelin-1 derived from ECs stimulates the expression of adhesion molecules and VSMCs proliferation in a paracrine manner. Our results provide direct evidence that ET-1 derived from ECs is a major contributor to the process of vascular remodelling in the model of flow cessation, greater than that of ET-1 derived from other cells.

The use of either selective or non-selective ET receptor antagonist has been approved for diseases with vascular inflammation, in particular, pulmonary hypertension. However, learning from the incidence of several side effects and the failure of the trials for congestive heart failure, probably due to the non-selective blockade of ET-1 in other cells, the result of our study suggests for a more efficient design in ET receptor antagonist with a targeted inhibition of ET-1 signalling, specifically in ECs.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgement

We thank Dr Cheryl E. Gariety for critically reading this manuscript; and Dr Naoto Sasaki and Dr Masakazu Shinhara for discussion on carotid ligation model.

Conflict of interest: none declared.

Funding

This study was supported in part by Grant-in-Aid for Scientific Research (C) to N.E. from Japan Society for the Promotion of Science (JSP).

References

15. Barton M, Haudenschild CC, d’Lusco LV, Shaw S, Munter K, Luscher TF. Endothelin ETA receptor blockade restores NO-mediated endothelial...


34. Shah PK. Inflammation, neointimal hyperplasia, and restenosis: as the leukocytes roll, the arteries thicken. Circulation 2003;107:2175–2177.


