Nectin-Like Molecule-5 Regulates Intimal Thickening After Carotid Artery Ligation in Mice

Fumie Kureha, Seimi Satomi-Kobayashi, Yoshiaki Kubo, Mitsuo Kinugasa, Tatsuro Ishida, Yoshimi Takai, Ken-ichi Hirata, Yoshiyuki Rikitake

Objective—Intimal thickening is considered to result from the dedifferentiation of medial smooth muscle cells (SMCs) from a contractile to a synthetic phenotype, and their subsequent migration and proliferation. It is unknown whether nectin-like molecule (Necl)-5, which is overexpressed in cancer cells, is involved in intimal thickening.

Approach and Results—Necl-5 was upregulated in mouse carotid artery after ligation. Compared with wild-type mice, intimal thickening after carotid artery ligation was milder in Necl-5 knockout mice. In vitro, the expression levels of SMC differentiation markers were higher, whereas the expression level of an SMC dedifferentiation marker was lower, in Necl-5 knockout mouse aortic SMCs (MASMCs) compared with wild-type MASMCs. The migration, proliferation, and extracellular signal–regulated kinase activity in response to serum were decreased in Necl-5 knockout MASMCs compared with wild-type MASMCs. In wild-type MASMCs, inhibition of extracellular signal–regulated kinase activity increased the expression levels of SMC differentiation markers and decreased their migration and proliferation in response to serum.

Conclusions—The present findings indicate that Necl-5 plays a role in the formation of intimal thickening after carotid artery ligation by regulating dedifferentiation, migration, and proliferation of SMCs in an extracellular signal–regulated kinase-dependent manner. Our results suggest that Necl-5 may represent a potential therapeutic target to limit intimal thickening after vascular injury. (Arterioscler Thromb Vasc Biol. 2013;33:1206-1211.)

Key Words: atherosclerosis ■ cell adhesion molecules ■ differentiation ■ intimal thickening ■ smooth muscle cells

Formation of intimal thickening is observed in arteries with atherosclerosis or restenosis after angioplasty or stent application.1 Although intimal thickening has been extensively studied at the molecular level, there is no effective preventive therapy or treatment for this disease. During the process of intimal thickening formation, one of the characteristic changes is the accumulation of smooth muscle cells (SMCs) within the intima. The proliferative response of SMCs has an important role in recovery from injury as well as atherosclerosis.2 It is believed that medial SMCs migrate into the subendothelial space, proliferate, and synthesize extracellular matrix proteins. In this process, SMCs switch from a contractile (differentiated) to a synthetic (dedifferentiated) phenotype, which augments their migration from the media to the intima and proliferation.3,4 Phenotypic modulation of SMCs plays a key role in a number of diseases, including atherosclerosis, restenosis, hypertension, and aneurysm.5 Various factors such as serum response factor complex,6 extracellular matrix components,7 platelet-derived growth factor,8 transforming growth factor-β,9 Notch family members,10 and reactive oxygen species11 are involved in this modulation. Differentiated SMCs express a variety of contractile proteins, including α smooth muscle actin (αSMA), smooth muscle 22 α (SM22α), and smooth muscle myosin heavy chain (SM-MHC).12 When SMCs are switched from a contractile to a synthetic phenotype, these differentiation markers are downregulated, whereas dedifferentiation markers such as smooth muscle embryonic (SMemb)/nonmuscle myosin heavy chain B are upregulated.13 Nectin-like molecules constitute a family of immunoglobulin-like cell adhesion molecules that contains the 5 members, Necl-1 to Necl-5.14 In addition to cell–cell adhesions, nectin-like molecules regulate cell migration, proliferation, and survival.14,15 Necl-5 was originally identified as human poliovirus receptor, also termed CD155,16,17 and as rodent Tage4, which was reported to be overexpressed in rodent colon carcinoma.18,19 In rodents, expression of Necl-5 is ubiquitous, but its level of expression is extremely low in most adult organs.20,21 However, it becomes upregulated in the developing or regenerating liver22,23 and cancer cells.24,25 Recently, we reported that Necl-5 is upregulated in the mouse hindlimb after ischemia and in cultured human endothelial cells exposed to hypoxia.26 Necl-5 forms a complex with integrin αβ3 and growth factor receptors, such as platelet-derived growth factor receptor and vascular endothelial growth factor receptor, to enhance the migration and proliferation of fibroblasts and endothelial cells.26,27 In a previous study

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on endothelial cells, we noticed that Necl-5 is expressed in vascular SMCs in mice. However, the role of Necl-5 in vascular SMCs is unknown. In the present study, we examined the role of Necl-5 in intimal thickening and its mode of action in the differentiation, migration, and proliferation of SMCs.

Materials and Methods
Materials and Methods are available in the online-only Supplement. All animal experiments were approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimental Regulations. Eight-week-old male Necl-5 knockout (KO) mice (50% 129/Sv, 25%, C57BL/6, and 25%, DBA) or age-matched wild-type (WT) mice on the same background were subjected to carotid artery ligation as described previously. Quantitative real-time polymerase chain reaction, immunofluorescence microscopy, and bromodeoxyuridine (BrdU) incorporation assay were performed. Mouse aortic SMCs (MASMCs) were isolated from the aortas of 8-week-old WT and Necl-5 KO mice as described previously. Western blotting and cell migration and proliferation assays were performed as described previously.

Results
Upregulation of Necl-5 in the Mouse Carotid Artery After Ligation
We examined the expression level of the Necl-5 mRNA in mouse carotid arteries. It was increased in ligated carotid arteries 3 and 7 days after ligation compared with unligated carotid arteries (Figure 1A). We then analyzed the localization of Necl-5 in carotid arteries. The immunofluorescence signal for Necl-5 was hardly detected in unligated carotid arteries, whereas it was more strongly observed in the thickened neointima formed after carotid artery ligation (Figure 1B). In high-magnification images of ligated carotid arteries, the signal for Necl-5 was strongly observed in neointimal cells positive for the dedifferentiation marker SMemb (Figure 1B). In addition, the signal for Necl-5 was weakly observed in medial cells positive for the differentiation marker αSMA (Figure 1B). These findings indicate that Necl-5 is upregulated in mouse carotid arteries after ligation and expressed in dedifferentiated SMCs in the thickened neointima.

Decreased Intimal Thickening After Carotid Artery Ligation in Necl-5 KO Mice
To examine the role of Necl-5 in intimal thickening, we compared the intimal thickening between WT and Necl-5 KO mice. Constrictive remodeling and intimal thickening were observed in the ligated carotid arteries of both WT and Necl-5 KO mice, but were decreased in Necl-5 KO mice compared with WT mice (Figure 2A and 2B). As a result, the lumen of the ligated carotid arteries was larger in Necl-5 KO mice than in WT mice (Figure 2B). However, there were no differences in the internal elastic lamina- or external elastic lamina-surrounding areas between these 2 types of mice (Figure I in the online-only Data Supplement). Intima/media ratio was significantly decreased in Necl-5 KO mice compared with WT mice (Figure 2C). Collectively, these findings indicate that Necl-5 augments intimal thickening after carotid artery ligation in mice.

Decreased BrdU Incorporation in the Carotid Artery After Ligation in Necl-5 KO Mice
Because intimal thickening is attributed largely to SMC proliferation, we analyzed SMC proliferation by BrdU incorporation in vivo. BrdU incorporation was observed in the intima and media of the ligated carotid arteries in both WT and Necl-5 KO mice, but was decreased in the intima and media in Necl-5 KO mice compared with WT mice (Figure 3A and 3B). These findings indicate that Necl-5 augments SMC proliferation after carotid artery ligation in mice.

Decreased Dedifferentiation, Migration, and Proliferation of Necl-5 KO MASMCs
To clarify the mechanism by which Necl-5 augments intimal thickening after carotid artery ligation in mice, we isolated MASMCs from WT and Necl-5 KO mice. We first analyzed their dedifferentiation in vitro. Compared with WT MASMCs, αSMA, SM22α, and SM-MHC, differentiation markers for SMCs, were significantly upregulated, whereas SMemb, a dedifferentiation marker, was downregulated in Necl-5 KO MASMCs (Figure 4A). Consistent with this, the expression of the differentiation and dedifferentiation marker proteins was changed as estimated by Western blotting (Figure 4B). We then analyzed migration and proliferation of MASMCs. The migration of Necl-5 KO MASMCs in the presence of 15% fetal bovine serum (FBS), but not 0.4% FBS, was markedly decreased compared with WT MASMCs (Figure 4C). The proliferation of Necl-5 KO MASMCs in the presence of 15% FBS was decreased compared with WT MASMCs (Figure 4D). Notably, the number of WT MASMCs in cultures with 0.4% FBS remained unchanged, whereas the number of Necl-5 KO MASMCs decreased in a time-dependent manner (Figure 4D), suggesting that Necl-5 plays a key role in cell survival. There were no significant differences in the numbers of adherent cells on various matrices between WT MASMCs and Necl-5 KO MASMCs (Figure II in the online-only Data Supplement). Taken together, these findings indicate that Necl-5 augments the differentiation, migration, and proliferation of SMCs.

Involvement of ERK Activity in the Dedifferentiation, Migration, and Proliferation of MASMCs
We then examined whether extracellular signal-regulated kinase (ERK) is involved in the differentiation, migration, and proliferation of SMCs. In WT MASMCs cultured in 15% FBS-containing media, the mitogen-activated protein/ERK kinase inhibitor U0126 (1 μmol/L) inhibited ERK activity (Figure 5A). U0126 (1 μmol/L) increased the expression of αSMA, SM22α, and SM-MHC, differentiation markers, in WT MASMCs (Figure 5B and 5C). This inhibitor decreased the 15% FBS–induced migration (Figure 5D) and proliferation (Figure 5E) of WT MASMCs. These findings indicate that ERK activity is critical for the differentiation, migration, and proliferation of SMCs.

Decreased ERK Activity in Necl-5 KO MASMCs
We then examined whether Necl-5 regulates ERK activity in SMCs. The basal level of ERK phosphorylation was decreased in Necl-5 KO MASMCs compared with WT MASMCs. The ERK phosphorylation level was increased by incubation with
15% FBS in both WT and Necl-5 KO MASMCs, but was lower in Necl-5 KO MASMCs compared with WT MASMCs (Figure 6A and 6B). These findings indicate that Necl-5 regulates ERK activity in MASMCs.

**Discussion**

Necl-5 is involved in various pathophysiological processes, given that it is overexpressed in the regenerating liver after injury, hindlimbs after femoral artery ligation, and cancer cells. The expression of Necl-5 is regulated by the Ras–ERK–activator protein 1 pathway. Because Necl-5 regulates the activation of the Ras–ERK–activator protein 1 pathway, it further enhances its own expression by activation of this signaling pathway in an auto-amplification fashion. In the present study, we found that Necl-5 was upregulated in dedifferentiated SMCs that were transmigrated from the media to the neointima after carotid artery ligation. ERK is reported to be activated in neointimal SMCs after carotid artery ligation. It has been suggested that, besides the altered shear stress conditions after ligation, other factors, such as oxidized lipids, growth factors, and cytokines, might concur to ERK activation. Indeed, we have shown here that Necl-5 regulates ERK activity in cultured MASMCs. Thus, ERK activation after carotid artery ligation probably promotes the upregulation of Necl-5 in SMCs.

We have shown here that Necl-5 is involved in intimal thickening. The lumen areas and intima/media ratios of the ligated arteries were significantly larger and lower, respectively, in Necl-5 KO mice compared with WT mice. The cell proliferation within the arterial wall after ligation evaluated by BrdU incorporation was decreased in Necl-5 KO mice. Consistent with this, the proliferation of Necl-5 KO MASMCs was
decreased compared with WT MASMCs. In addition, comparable cell migration was observed in the presence of 0.4% FBS between WT and Necl-5 KO MASMCs. However, significant impairment of Necl-5 KO MASMC migration was observed in the presence of 15% FBS. Collectively, these findings suggest that the decreased proliferation and migration caused by the absence of Necl-5 contribute to the reduced intimal thickening. There were no abnormalities in vascular development and arteriogenesis in Necl-5 KO mice (data not shown), despite the decreased proliferation and migration of Necl-5 KO MASMCs. Further studies are needed to clarify the compensatory mechanism for the absence of Necl-5 during ontogenesis.

We have shown here that Necl-5 regulates the differentiation of SMCs, because differentiation markers, such as αSMA, SM22α, and SM-MHC, were upregulated, whereas SMemb, a dedifferentiation marker, was downregulated in the absence of Necl-5. Because ERK activity regulates the expression of αSMA, SM22α, and SM-MHC, it is likely that the absence of Necl-5 decreased ERK activity, leading to the downregulation of these markers. However, the effect of the mitogen-activated protein/ERK kinase inhibitor was lower than that in the absence of Necl-5. Therefore, signaling pathways other than the ERK pathway may play a role in the effects of Necl-5. Furthermore, inhibition of ERK activity did not decrease the expression of SMemb (data not shown), indicating that an ERK-independent mechanism is involved in SMemb expression. SMemb expression is regulated by the transcription factor, Krüppel-like factor 5/basic transcriptional element binding protein-2.34 The expression and activity of Krüppel-like factor 5 are regulated by multiple signaling pathways, including Ras/ERK,35 protein kinase C,36 and transforming growth factor-β.37 It is not currently known whether Necl-5 is associated with protein kinase C or transforming growth factor-β signaling.

Necl-5 enhances the migration and proliferation of NIH3T3 cells27 and endothelial cells.26 Consistent with these previous studies, Necl-5 enhanced the migration and proliferation of SMCs. In NIH3T3 cells, Necl-5 interacts with sprouty2, a protein that inhibits the Ras/ERK signaling pathway,38,39 and prevents the tyrosine phosphorylation and activation of sprouty2.40 In the absence of Necl-5, sprouty2 is free from Necl-5, which results in its activation to inhibit the Ras/ERK signaling pathway for cell proliferation. It was reported that sprouty2 inhibits the proliferation and migration of SMCs, and decreases the growth of the neointima and cellular proliferation in a rat carotid artery balloon injury model.41 It is noteworthy that sprouty2 is upregulated in neointimal SMCs.41 We did not investigate whether sprouty2 interacts with Necl-5 in SMCs, but we assume that the increased Necl-5 expression...
may suppress the inhibitory actions of sprouty2 in neointimal SMCs, resulting in neointima formation despite the increase in sprouty2 expression.

Intimal thickening is the most critical cause of restenosis, particularly after coronary angioplasty and coronary artery bypass surgery.1 The results of the present study implicate Necl-5 as a therapeutic target for intimal thickening that contributes to restenosis after coronary intervention. For example, an anti–Necl-5 mAb42 may have the potential to prevent intimal thickening, although further extensive studies are required to determine the clinical utility of direct Necl-5 inhibition.

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Disclosures
None.

References
Intimal thickening is an important phenomenon in the progression of atherosclerosis. Dedifferentiation of smooth muscle cells from the contractile phenotype to the synthetic phenotype as well as succeeding smooth muscle cell migration and proliferation are key events in the formation of intimal thickening. The identification and characterization of factors controlling these events are crucial for preventing the formation of intimal thickening and consequently atherosclerosis. This is the first report in which the role of necdin-like molecule-5, a member of the nectin/nectin-like molecule family proteins, in intimal thickening and smooth muscle cell function is examined. Nectin-like molecule-5 regulates intimal thickening after carotid artery ligation in mice and differentiates, migration, and proliferation of cultured smooth muscle cells. These findings suggest that nectin-like molecule-5 may represent a potential therapeutic target to limit intimal thickening after vascular injury.
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**Online Figure I.** No differences in the areas defined by the IEL and EEL between WT and Necl-5 KO mice. The areas defined by the IEL and EEL in unligated or ligated carotid arteries are shown ($n = 14$).
**Online Figure II.** No effects of Necl-5 deficiency on cell adhesion to various matrices. Ninety-six well plates were precoated with 20 µg/mL collagen, 1 µg/mL fibronectin, 3 µg/mL vitronectin, or 50 µg/mL laminin overnight at 4°C. MASMCs were suspended in DMEM supplemented with 0.4% FBS at a density of 5 × 10⁵ cells/ml. A 100 µL aliquot of cell suspension was applied to each well and then incubated for 1 hour at 37°C in 5% CO₂. After incubation, the unattached cells and residual buffer were removed. The remaining cells were stained with 0.5% crystal violet and lysed in 100 µL of 10% acetic acid. The optical density of the crystal violet solutions at 595 nm was measured. Each value represents the mean ± SEM of 6 wells. The results shown are representative of three independent experiments.
Materials and Methods
Antibodies and Reagents
Rat anti-Necl-5 monoclonal antibody (mAb) (R&D Systems, Inc., Minneapolis, MN), rabbit anti-αSMA polyclonal antibody (pAb), rabbit anti-SMemb pAb (GeneTex, Inc., Irvine, CA), goat anti-SM22α pAb (Abnova, Taipei, Taiwan), rabbit anti-bromodeoxyuridine (BrdU) pAb (Abbiotec, LCC, San Diego, CA), mouse anti-GAPDH mAb (Fitzgerald Industries International Inc., North Acton, MA), rabbit anti-phospho-ERK 1/2 pAb, and rabbit anti-ERK pAb (Cell Signaling Technology, Danvers, MA) were purchased from the indicated suppliers. U0126, a mitogen-activated protein/ERK kinase (MEK) inhibitor, was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Carotid Artery Ligation
All animal experiments were approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimental Regulations. Eight-week-old male Necl-5 knockout (KO) mice (50% 129/Sv, 25%, C57BL/6, and 25%, DBA)\(^1\) (\(n = 20\); 14 for intimal thickening and 6 for BrdU incorporation assay) or age-matched wild-type (WT) mice on the same background (\(n = 73\); 48 for mRNA extraction, 5 for immunofluorescence microscopy, 14 for intimal thickening, and 6 for BrdU incorporation assay) were subjected to carotid artery ligation as described previously.\(^2\) Briefly, the mice were anesthetized intraperitoneally with 2.5% 2,2,2-tribromoethanol (1.6 mL/100 g). The right common carotid artery was exposed through a midline cervical incision and ligated at the bifurcation with 6-0 silk. Immunostaining of Necl-5, αSMA, SMemb, and CD31 as well as BrdU incorporation were assessed at 14 days after ligation, and intimal thickening was assessed at 28 days after ligation. After anesthesia, the mice were transcardially perfused with 2% paraformaldehyde. The common carotid arteries were removed, post-fixed in 2% paraformaldehyde for 2 hours, stored in 20% sucrose for 2 hours, and then maintained in 25% sucrose overnight. Freezing microtome sections (6 μm) were collected every 50-μm interval (0.5- to 3.0-mm segments proximal to the ligation site) and stored at −20°C until processing for each assay. Intimal thickening was analyzed using ImageJ software (NIH). All serial sections (approximately 50 sections) were divided into 5 nearly equal groups, and 2 sections were selected from each group. Luminal area was measured by tracing along the luminal surface. The medial area was calculated by subtracting the area defined by the internal elastic lamina (IEL) from...
that defined by the external elastic lamina (EEL), and the intimal area was calculated by subtracting the luminal area from the area defined by the IEL. The area ratios of the intima to the media (Intima/Media ratio) were calculated. Thus, the section with the maximum intima/media ratio selected from the 10 sections was analyzed for each mouse.

Quantitative Real-time Polymerase Chain Reaction (qPCR)
Total mRNAs were extracted from WT mouse carotid arteries or WT and Necl-5 KO mouse aortic SMCs (MASMCs) using TRIzol Reagent (Invitrogen, Carlsbad, CA). mRNAs extracted from 4 mouse carotid arteries were combined in a single sample. A ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) was used for reverse-transcription. qPCR was performed using a 7500 real-time PCE system (Applied Biosystems, Carlsbad, CA) with Takara SYBR® Premix EX Taq II (Perfect real time) (Takara Bio, Otsu, Japan). Primers for Necl-5, αSMA, SM22α, SM-MHC, SMemb, and ribosomal protein S18 were purchased from Takara Bio. Ribosomal protein S18 was used for normalization, and the comparative threshold method was used to assess the relative abundance of the target mRNAs.

Immunofluorescence Microscopy
Sections were stained with the indicated Abs and then with appropriate fluorophore-conjugated secondary Abs. The fluorescent signals were visualized with a confocal laser scanning microscope (LSM700; Carl Zeiss, Jena, Germany).

BrdU Incorporation Assay
Mice (n = 6, each genotype) were injected intraperitoneally with BrdU (Roche, Basel, Switzerland; 10 mmol/L; 1 mL/100 g) overnight prior to harvesting the carotid arteries. For the detection of BrdU-labeled nuclei in sections, the following DNA denaturation steps preceded incubation with the anti-BrdU pAb: incubation in 50% formamide/2× SSC (0.3 mol/L NaCl, 0.03 mol/L sodium citrate) at 65°C for 2 hours; rinse in 2× SSC for 5 minutes; incubation in 2 mol/L HCl at 37°C for 30 minutes; and rinse in 0.1 mol/L boric acid (pH 8.5) for 10 minutes.

Cell Culture
MASMCs were isolated from the aortas of 8-week-old WT and Necl-5 KO mice as described previously.² Cells between passages 2 and 7 were plated on non-coated dishes and plates, cultured
in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum (FBS), glutamine, penicillin, streptomycin, fungizone and non-essential amino acids, and used for experiments.

**Western Blotting**

Western blotting was performed according to standard methods, as described previously. Densitometric analysis was performed using ImageJ software (NIH).

**Assays for Cell Migration and Proliferation**

Assays for cell migration and proliferation were performed essentially as described previously. Cell migration assays were performed using Falcon cell culture inserts with PET membranes (8.0-µm pores; Becton Dickinson Labware, Franklin Lakes, NJ) precoated with 0.1% gelatin overnight at 4°C. MASMCs were plated at a density of $5 \times 10^4$ cells/insert. The cells were incubated at 37°C for 4 hours in DMEM supplemented with 0.4% or 15% FBS. The numbers of migrated cells in five randomly chosen fields per filter were counted by microscopic examination. For cell proliferation, MASMCs were plated in 96-well plates at $5 \times 10^3$ cells/well and cultured in DMEM supplemented with 0.4% or 15% FBS at 37°C under 5% CO$_2$. The numbers of cells were quantified by crystal violet staining.

**Statistical Analysis**

All experiments were performed at least three times, and the results are expressed as means ± SEM. Differences between groups were compared using Student’s $t$-test. Values of $P < 0.05$ were considered significant.

**References**

