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Objective—Light-type caldesmon (*l*-CaD) is a potent cytostatic and antiangiogenic protein that regulates cell growth and survival via modulation of the cell shape and cytoskeleton. The aim of this study is to explore the potential value of *l*-CaD for use as a cytostatic agent to inhibit neointimal formation after angioplasty by suppressing vascular smooth muscle cell (VSMC) growth and migration.

Methods and Results—We tested the cytostatic function of *l*-CaD in cultured VSMCs using assays for apoptosis, cell proliferation, and migration, and evaluated the expression pattern of relevant signaling proteins (focal adhesion kinase [FAK] and mitogen-activated protein kinases) in VSMCs. Transfection of adenoviral vector encoding *l*-CaD (Ad-*l*-CaD) resulted in progressive loss of actin stress fibers and cell retraction. Enzyme-linked immunosorbent assay demonstrated that Ad-*l*-CaD transfection increased the apoptosis rate by 75% and reduced BrdU uptake by 49%. Furthermore, transfection of Ad-*l*-CaD inhibited migration of VSMCs induced by platelet-derived growth factor-BB (PDGF) by 36% ($P<0.05$). Immunoblotting analysis revealed that *l*-CaD overexpression reduced PDGF-induced phosphorylation of both FAK and extracellular signal regulated-kinase (ERK). In balloon-injured rat carotid arteries, Ad-*l*-CaD transfection inhibited neointimal formation by 37% ($P<0.05$) without delaying re-endothelialization at 14 days.

Conclusions—Overexpression of *l*-CaD suppressed cell growth and survival in VSMCs and inhibited neointimal formation after experimental angioplasty, partly by regulating the cytoskeletal tension-FAK-ERK axis. (***Arterioscler Thromb Vasc Biol*. 2006;26:2231-2237.)**

Key Words: animal models of human disease ■ cell biology/structural biology ■ gene therapy ■ restenosis ■ smooth muscle proliferation and differentiation

Progress in percutaneous coronary interventions such as the development of drug-eluting stents (DES) and antithrombus therapies has dramatically decreased the mortality and morbidity in patients with coronary artery disease.[1–3] Many clinical trials of DES implantation in coronary arteries have resulted in fairly low restenosis rates of <5%.[4,5] In the pre-DES era, the strategies used to reduce restenosis after stent implantation (in-stent restenosis) can be categorized into 3 groups according to the underlying processes: (1) cytostatic strategies, which focus on inhibition of cell cycle entry (eg, retinoblastoma protein,[6] cyclin-dependent kinase inhibitor protein p21[7]); (2) cytotoxic strategies, which induce the death of cells that enter the cell cycle (eg, thymidine kinase isozyme derived from herpes simplex virus,[8] and cytosine deaminase[9]); and (3) paracrine strategies, which affect cells at several stages in the progression of restenosis, such as antithrombosis and re-endothelialization, and inhibits vascular smooth muscle cell (VSMC) proliferation (eg, endothelial nitric oxide synthase,[10] and prostacyclin synthase,[11,12]). At present, the cytostatic agents rapamycin and paclitaxel, both of which inhibit cell cycle progression at the late G1 and M phases, respectively, are the most clinically proven and predominantly used drugs in DES.[4,5]

Paclitaxel was originally developed as an anticancer drug based on its ability to interfere with microtubule polymerization, and thereby prevent mitosis; however, it also suppresses cancer cell migration based on its effects on the interphase cytoskeleton.[13] Previous studies have revealed that cell growth, migration, and apoptosis also can be controlled by modifying the structure or tension-generating capacity of the actin cytoskeleton in various cell types including all of the cells of the vascular wall (VSMCs, endothelial cells, and fibroblasts).[14–16]

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From Nagoya University Graduate School of Medicine (K.Y., Y.N., R.K., R.M., Y.O., T.K., K.O., T.M.), Department of Cardiology; Nagoya University School of Medicine (Y.N., M.I.), Department of Medical Science of Proteases, Showa, Nagoya, Japan; Komaki Municipal Hospital, Department of Cardiology (H.I.), Komaki, Aichi, Japan; Vascular Biology Program (D.E.I.), Departments of Pathology and Surgery, Children's Hospital & Harvard Medical School, Boston, Mass.

Correspondence to Yasushi Numaguchi, Nagoya University Graduate School of Medicine, Department of Cardiology; and Nagoya University School of Medicine, Department of Medical Science of Proteases, 65 Tsurumai, Showa, Nagoya 466-8550, Japan. E-mail numa2@med.nagoya-u.ac.jp

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Caldesmon (CaD) is one of the proteins that regulate the actin cytoskeleton. It is found within the contractile apparatus, and binds to its various component proteins, including actin, myosin, tropomyosin, and calmodulin.[17–20] When CaD binds to actin, it inhibits the ATPase activity of actomyosin in a calcium-and calmodulin-dependent manner.[21] CaD has 2 isoforms: high- (*h*-CaD) and low-molecular-weight (*l*-CaD) types. *l*-CaD is the smaller of these 2 isoforms, and shares functionally important domains with the larger CaD (*h*-CAD) except for the central domain.[22,23] Past in vitro studies have shown that transfection of cells with high levels of *l*-CaD results in loss of actin stress fibers, disruption of focal adhesions (FAs), and cell retraction in cultured fibroblasts,[24] Moreover, these same CaD-induced cytoskeletal alterations result in reduced cell growth and enhanced apoptosis in capillary endothelial cells.[25] In this study, we explored whether *l*-CaD could exhibit cytostatic activity using assays for apoptosis, cell proliferation, and migration in cultured VSMCs. Furthermore, to determine the potential value of *l*-CaD as a therapeutic for vascular restenosis, we assessed its inhibitory effects on neointimal formation in a balloon-injured artery model by overexpressing *l*-CaD in vivo using an adenoviral vector.

Methods

Construction of Recombinant Adenovirus Vectors

The replication-incompetent recombinant adenovirus vector used in this study was constructed as described previously.[25] Briefly, green fluorescent protein (GFP)-tagged cDNA containing the full-length rat nonmuscle CaD (*l*-CaD) gene was inserted into the adenoviral DNA construct provided by BD Biosciences Clontech (Palo Alto, Calif). Virus particles were purified and concentrated with a titering kit (BD Biosciences Clontech) and stored at −80°C before use in experiments.

Cell Culture for VSMCs and Endothelial Cells

Human aortic VSMCs were purchased from Kurabo (Osaka, Japan) and cultured with Hu-Media SG2 (Kurabo) containing 5% fetal bovine serum, antibiotics (50 μg/mL gentamycin, 50 ng/mL amphotericin B), human basic fibroblast growth factor (2 ng/mL), human epidermal growth factor (0.5 ng/mL), and insulin (5 μg/mL).[26] VSMCs at passages 4 to 7 were used in the experiments. VSMCs were grown into 70% to 80% confluence and then made quiescent by 1% fetal bovine serum media (Hu-Media SD2, Kurabo) containing antibiotics (50 μg/mL gentamycin, 50 ng/mL amphotericin B) and heparin (30 μg/mL) for 48 hours before experiments.

Human umbilical vascular endothelial cells (HUVECs) that were kindly supplied by Dr Keiji Naruse (Okayama University) were cultured in Hu-Media EG2 (Kurabo) containing 2% fetal bovine serum with the antibiotics listed and EC supplements (Kurabo) as previously described.[25] HUVECs at passage 2 or 3 were used in the experiments.

Drug Treatment and Adenovirus-Mediated Gene Transfer to VSMCs and ECs

To observe cytoskeletal changes within VSMCs, cells were treated with paclitaxel (1 μmol/L, Wako, Osaka, Japan) for 24 hours, rinsed with fresh medium, and observed using an epifluorescence microscope (Olympus).

In vitro gene transfer to VSMCs was performed by incubation with Ad-GFP or Ad-GFP-*l*-CaD at a multiplicity of infection of 100 in Hu-Media SD2 at 37°C, 5% CO_2 and 95% air, with 1 μg/mL doxycycline. Cells were then cultured for 36 to 48 hours. The expressions of GFP and GFP-*l*-CaD were induced by culturing the cells in the same low-serum medium without doxycycline. VSMCs were quiescent for 24 to 48 hours before being used in the following experiments. HUVECs were transfected in a similar manner and incubated for 48 hours. To visualize the cytoskeletal alteration at specific times, cells were fixed, incubated with Alexa Fluor™ 488 or 594 conjugated with phalloidin (Invitrogen, Carlsbad, Calif) and 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen), and observed using an epifluorescence microscope.[25]

Cell Proliferation Assay and Apoptosis Assay

VSMCs (1×10^4 cells/100 μL for the cell proliferation assay, 2×10^4 cells/100 μL for the DNA fragmentation assay) were exposed to the adenovirus in 96-well plates under the conditions described above. At 24 hours after doxycycline removal, 100 μL of high-serum medium was added and cells were cultured for an additional 24 hours to induce the cells to reenter the cell cycle.

The proliferative activity of adenovirus-transfected VSMCs were estimated from the uptake of 5-bromo-2′deoxyuridine (BrdU). Ten micromolar BrdU was added to each well and the cells were incubated for another 2 hours. The cells were then fixed and nuclear incorporation of BrdU was measured using a cell proliferation enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostics, Mannheim, Germany). To compare the suppressive effects of *l*-CaD on the proliferation of VSMCs and ECs, the WST-1 assay (Takara) was performed at 48 and 72 hours after GFP or GFP-*l*-CaD gene transfection (multiplicity of infection of 100) according to the manufacturer's protocol.

To assess the effect of *l*-CaD overexpression on apoptosis, cytoplasmic DNA fragmentation (an indicator of apoptosis) was detected by using the Cell Death Detection ELISA^{PLUS} (Roche Diagnostics). Both ELISA assays were performed in accordance with the manufacturer's instructions. Each measurement was performed in triplicate, and the results are presented as percentages relative to untreated control cultures. Furthermore, we evaluated the levels of protein expression of antiapoptotic signaling for bcl-2 in VSMCs at 24 hours after GFP or GFP-*l*-CaD gene transfection (multiplicity of infection of 100). Similarly, a caspase-3 assay (BioVision, Mountain View, Calif) was performed according to the manufacturer's protocol.[27]

Migration Assay

The migration assay was performed with Transwell (Corning, Acton, Mass) 24-well tissue culture plates comprising a polycarbonate membrane with 8-μm pores. The inner chamber membrane was coated with 50 μL of Matrigel solution (50 μg/mL; BD BioScience, San Jose, Calif) at 4°C overnight to avoid polymerization, and then rinsed with DMEM. VSMCs were then seeded on the inner chamber of the Transwell plate at a concentration of 1×10^5 cells/100 μL. The inner chamber was placed into the outer chamber filled with 600 μL of serum-free DMEM, which contained recombinant human platelet-derived growth factor (PDGF)-BB (10 ng/mL), and incubated for 6 hours at 37°C. The cells that migrated onto the outer surface of the membrane were fixed with cold methanol and 4% paraformaldehyde, and stained by the May-Giemsa method. The number of migrated cells was counted in 4 to 6 randomly chosen fields of the duplicated chambers at a magnification of ×200 for each sample.[28]

Immunoblotting Analysis

VSMCs were cultured and exposed to the adenovirus in a six-well plate. After 48 hours of quiescence, PDGF-BB (10 ng/mL; PeproTech, London, UK) was added and cells were incubated for a further 30 minutes. VSMCs were then lysed in Laemmli Sample Buffer (Sigma, St. Louis, Mo), and the cell lysate was used for immunoblotting analysis as previously described.[29]

The following antibodies were used in this study: rabbit anti-FAK and anti-phospho FAK (Biosource, Camarillo, Calif), rabbit anti-p44/42 MAP kinase (ERK1/2) and anti-phospho p44/42 MAP kinase, rabbit anti-SAPK/JNK, mouse anti-phospho SAPK/JNK, rabbit anti-p38 MAP kinase, mouse anti-phospho p38 MAP kinase (Cell Signaling, Beverly, Mass), and mouse anti-bcl-2 (Sigma).[27]

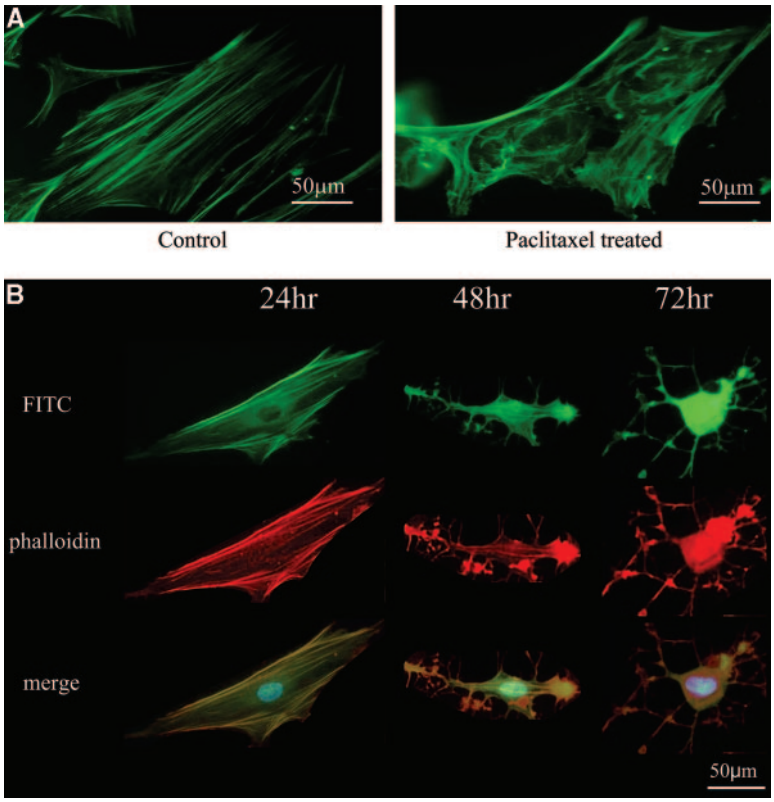

Figure 1. Cytoskeletal disruption by paclitaxel and *l*-caldesmon (*l*-CaD). Actin filaments were stained with Alexa Fluor™ 488/phalloidin. Paclitaxel treatment disrupted actin filaments in accordance with the disruption of microtubule networks (A). If *l*-CaD is overexpressed in vascular smooth muscle cells (VSMCs), these cells progressively lose attached area and the cell shape becomes dendric (B). Fluoresceinisothiocyanate (FITC): VSMCs observed through FITC filter; phalloidin: VSMCs stained with Alexa Fluoro™ 594/phalloidin; merge: merged images with DAPI counterstaining.

In Vivo Gene Transfer

Male Sprague-Dawley rats weighing 350 to 400 g were obtained from SLC (Nagoya, Japan), and cared for and used in accordance with the guidelines of the National Institutes of Health. After inflicting a balloon injury to the rat right carotid artery, genes were transferred into the arteries in vivo as described previously.[12,29,30] Ad-GFP-*l*-CaD (10^9 pfu/mL, 100 μL) with 100 μL of phosphate-buffered saline (ie, total volume of 200 μL) was used for transfection. The balloon-injured rats were divided into Ad-GFP and Ad-GFP-*l*-CaD groups (each n=6). The systolic blood pressure and heart rate were measured before and 14 days after balloon injury by the tail-cuff method (BP98A; Softron, Tokyo, Japan).[31]

Histological Assessment

At 14 days after injury and transfer, rats were killed and the carotid arteries were perfusion fixed with 4% paraformaldehyde and harvested. The excised carotid arteries were paraffin embedded, sectioned at 4 μm, and mounted on glass slides. The sections were stained with hematoxylin-eosin and the cross-sectional area of the blood vessel layers including the intimal and medial areas were quantified using National Institutes of Health Image (by Wayne Rasband, National Institutes of Health). The morphometric parameters of the lumen area (LA), neointimal area (IA), medial area (MA), area surrounded by external elastic lamina (EEL), and intima/media (I/M) cross-section-area ratios were calculated in 4 separate cross-sections of each artery. The mean measurements were used to calculate the I/M ratios for each animal.[12,30] To assess re-endothelialization after *l*-CaD gene transduction, three sections from each of the control and GFP-*l*-CaD groups were immunostained with anti-von Willebrand factor antibody (DAKO), and the percentage of the inner-lumen circumference lined with newly regenerated endothelium was determined.[12,30]

Statistical Analysis

The data are presented mean±SEM values. Statistical analysis for multiple comparisons among the groups used 1-way ANOVA followed by the Bonferroni test. $P<0.05$ was considered statistically significant.

Results

Effects of Paclitaxel and l-CaD on Cell Shape

Paclitaxel, one of the drugs most commonly used in DES, produces its cytostatic effects by targeting cytoskeletal microtubules. Given that disruption of the actin cytoskeleton has been shown to have potent effects on cell behavior, and the actin and microtubule cytoskeletons form a single mechanically integrated system, we explored whether paclitaxel treatment altered the distribution of actin filaments in cultured VSMCs. These studies confirmed that disruption of microtubule network was accompanied by disassembly of normally well-developed actin stress fibers (Figure 1A) Transduction of these cells with an adenoviral vector encoding GFP-*l*-CaD resulted in significant changes in cell shape and cytoskeletal structure over time. The GFP-*l*-CaD colocalized with F-actin filaments at 24 hours, as detected by staining with Alexa Fluoro™ 594/phalloidin (Figure 1B). Continued expression of GFP-*l*-CaD resulted in the progressive disassembly of actin bundles, with only sparse small filaments containing both F-actin and GFP-*l*-CaD being present by 48 hours. By 72 hours, linear F-actin was lost, and the cells physically retracted so that they appeared dendritic in form. These changes in cell morphology were the same as those previously observed in other cell types.[17–25]

Effects on Cell Proliferation and Apoptosis

To determine whether *l*-CaD inhibited the proliferation of VSMCs, we examined the influence of *l*-CaD on cell prolif-

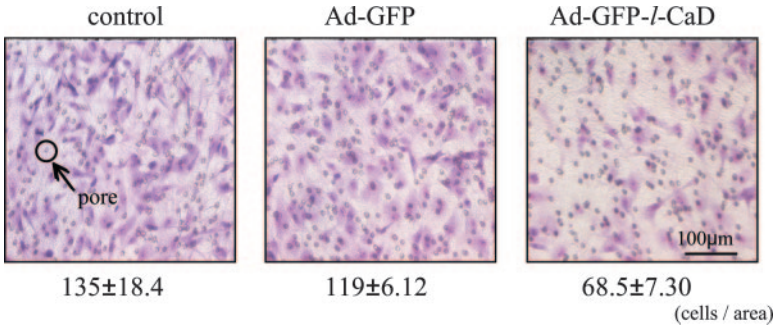

Figure 2. The effects of *l*-CaD overexpression on migration of VSMCs. Platelet-derived growth factor (PDGF)-BB–induced VSMC migration was suppressed by GFP-*l*-CaD overexpression ($P<0.05$ vs control and GFP groups). Representative images of migrated VSMCs. Circle shows micropore of the filter.

eration (DNA synthesis) by measuring the nuclear incorporation of BrdU. We found that GFP-*l*-CaD-gene transfer to VSMCs reduced BrdU uptake compared with control untreated cells or cells with GFP alone ($P<0.01$ versus control and GFP) (please see http://atvb.ahajournals.org). DNA fragmentation, a key hallmark of apoptosis, also was higher in GFP-*l*-CaD-transfected VSMCs relative to both controls ($P<0.01$) (please see http://atvb.ahajournals.org). In both assays, the difference between GFP-transfected cells and control cells was not significant.

Intracellular apoptosis was further evaluated based on analysis of expression of the antiapoptotic protein, bcl-2. Bcl-2 was found to be lower in GFP-*l*-CaD-transfected VSMCs than in control cells ($P<0.01$). (please see http://atvb.ahajournals.org) Independent studies confirmed that GFP-*l*-CaD-gene transfer to VSMCs promoted the activity of caspase-3, which is an important proteolytic initiator of cellular apoptosis ($P<0.01$ and 0.05 versus untreated and GFP-transfected alone, respectively).

Comparison of Effects on Cell Proliferation Between ECs and VSMCs

The WST-1 assay was used to compare the effects of *l*-CaD-protein overexpression on the proliferation of HUVECs versus VSMCs. We found that the proliferation rate decreased by similar levels in both cell types when analyzed 48 hours after induction of GFP-*l*-CaD-gene expression; however, in at 72 hours, the inhibitory effect of GFP-*l*-CaD was greater in HUVECs than in VSMCs ($P<0.01$). (Please see http://atvb.ahajournals.org)

Migration Assay Results

PDGF released from restenotic or atherosclerotic lesions acts as a chemoattractant and proliferative agent for VSMCs.[32] We therefore used PDGF-BB to evaluate the effect of GFP-*l*-CaD transfection on VSMC migration using the Transwell migration assay (Figure 2). We found that Ad-GFP-*l*-CaD gene transfer suppressed the PDGF-BB-induced migration of VSMCs, with a 42% reduction observed in the migration of Ad-GFP-*l*-CaD gene-transfected cells compared with Ad-GFP gene-transfected cells ($P<0.05$). In contrast, Ad-GFP transfection did not significantly affect PDGF-BB-induced migration (please see http://atvb.ahajournals.org).

Immunoblotting Analysis Results

We analyzed the effects of GFP-*l*-CaD on the key signaling molecules, FAK and ERK, because these kinases contribute to control of cell growth, migration and apoptosis, and they are regulated by tensional forces exerted on cell adhesions. Ad-GFP-*l*-CaD-transfected VSMCs exhibited 83% less phosphorylation of FAK than PDGF-stimulated nontransfected cells ($P<0.01$) (Figure 3A). Phosphorylation of ERK-1/2 in the VSMCs transfected with Ad-GFP-*l*-CaD also was reduced by 45% compared with control cells ($P<0.05$) (Figure 3B).However, Ad-GFP gene transfer did not significantly affect FAK or ERK1/2 phosphorylation. This effect appeared to be specific in that the phosphorylation of two other protein kinases, p38 MAPK and SAPK/JNK was not effected by transfection of Ad-GFP-*l*-CaD or Ad-GFP.

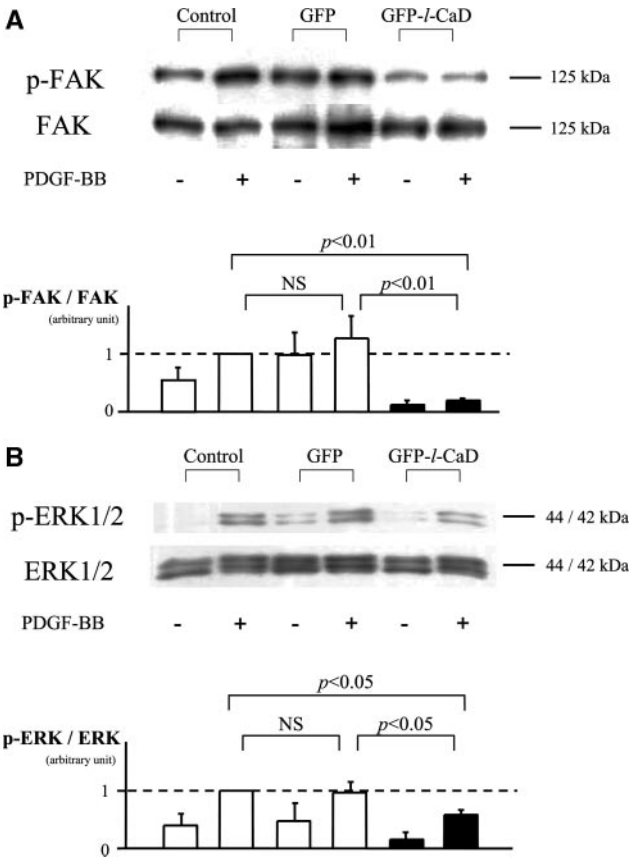

Figure 3. The effects of *l*-CaD overexpression on phosphorylation of focal adhesion kinase (FAK) and mitogen-activated protein kinases (MAPKs). GFP-*l*-CaD overexpression inhibited phosphorylation of FAK (A) and extracellular-signal-regulated kinase (ERK)-1/2 (B).

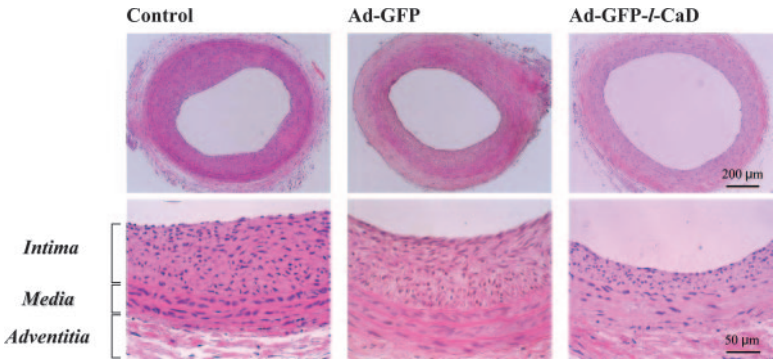

Figure 4. Cross-sections and morphometric analysis of carotid arteries 14 days after injury. Representative cross-sections of carotid arteries are shown. Arteries transfected with Ad-GFP-*l*-CaD shows diminished neointimal formation compared with vehicle-treated arteries.

Effects on Neointimal Formation After Balloon Injury of Rat Carotid Arteries

To investigate the effect of gene transfer on neointimal formation, we evaluated the morphometric analysis in each cross-section of arteries at 14 days after inflicting balloon injuries (Figure 4). The I/M ratio was 1.43±0.21 in the control group and 1.19±0.19 in the Ad-GFP group (n=6 in control group, n=4 in Ad-GFP group; NS), whereas the ratio was lower in the Ad-GFP-*l*-CaD group than in the other 2groups (0.69±0.12, n=6, $P<0.01$ versus control and Ad-GFP groups) The IA was also lower in the Ad-GFP-*l*-CaD group (0.11±0.03 mm^2) than in the control group (0.25±0.05 mm^2, $P<0.01$) and the Ad-GFP group (0.19±0.03 mm^2, $P<0.01$). In contrast, MA and EEL were not affected. The LA was larger in the Ad-GFP-*l*-CaD group (0.35±0.06 mm^2, $P<0.05$) than in the control group (0.22±0.13 mm^2) and the Ad-GFP group (0.24±0.13 mm^2). The coverage of the inner lumen by ECs did not differ between the control group (93±6%) and the Ad-GFP-*l*-CaD group (95±7%, *NS*) at 14 days after balloon injury, indicating that *l*-CaD-gene transfer did not affect regeneration of ECs or the homing of endothelial progenitor cells during the chronic phase after balloon injury.

Discussion

In the process of analyzing the mechanism of the cytostatic agents underlying the suppression of VSMC proliferation and neointimal formation, we found that paclitaxel could induce disruption not only of microtubule networks but also of actin filaments (Figure 1A). We demonstrated that cytoskeletal disruption by *l*-CaD could lead to inhibition of VSMC proliferation as well as of endothelial cells and fibroblasts via the ERK1/2 pathway. Furthermore, gene delivery of *l*-CaD inhibited neointimal formation in the rat balloon-injured artery model by 37%.

Many clinical trials have demonstrated that the cytostatic agents rapamycin and paclitaxel exhibit efficacy that is superior to other conventional devices or adjunctive therapy, and are the predominantly used as drugs in DES.[4,5] CaD is an actin-binding protein that inhibits the ATPase activity of actomyosin in a calcium- and-calmodulin-dependent manner. Previous studies have shown that the transfection of cells with high levels of nonmuscle CaD results in disruption of FAs, loss of actin stress fibers, and cell retraction in cultured fibroblasts,[24] and also reduces cell growth and induces apoptosis in capillary endothelial cells.[25] In the present study, we demonstrated that overexpression of *l*-CaD also suppresses VSMCs (in addition to the above cell types) and reduces intimal hyperplasia in balloon-injured rat carotid arteries.

FAs to the extracellular matrix of cells play a critical role in their proliferation, migration, and survival.[33,34] FAs act not only as the mechanical linkage that ties the cytoskeleton to bound integrins and the extracellular matrices, but also as biochemical signaling hubs for many regulatory pathways; therefore, regulation of the assembly and disassembly of FAs is a crucial mechanism for controlling cell function.[35] Integrin-mediated cell adhesion triggers tyrosine phosphorylation.[36,37] FAK is one of the protein tyrosine kinases localized in FAs.[35,38] FAK is rapidly phosphorylated at Tyr^{397} on integrin-mediated cell adhesion or growth factor receptor stimulation, and plays a crucial role in linking signals initiated by the integrins or growth-factor receptors to intracellular cytoskeletal and signaling proteins, thus controlling essential cellular processes such as growth, survival, migration, and differentiation.[39] In this study, the phosphorylation of FAK was significantly suppressed by overexpression of *l*-CaD even after PDGF treatment and after disruption of actin cytoskeleton in VSMCs. These results are consistent with previous studies showing that inhibitors of actin polymerization such as cytochalasin D and latrunculin B decrease FAK phosphorylation.[35] We considered that this suppressive effect of *l*-CaD overexpression on FAK phosphorylation sufficiently inhibits the signal transmission from FAs to the intracellular proteins to lead to inhibition of the proliferation and migration of VSMCs.

MAPKs, including ERK1/2, p38 MAPK, and SAPK/JNK, are a family of protein serine/threonine kinases. MAPKs are activated by a variety of stimuli, including growth factors, cytokines, hormones, and mechanical forces, and play the major role in cellular responses such as gene expression, cell proliferation, cell motility, cell survival and death, and cell differentiation.[40] ERK1/2 is reportedly induced after arterial injury in different animal models,[41] and the inhibitor of ERK1/2[42] and the gene transfer of the dominant-negative mutant of ERK1/2[43] can prevent VSMC proliferation and neointimal formation in balloon-injured arteries. ERK1/2 can also function as a CaD kinase in VSMCs.[44] Phosphorylation of CaD (at Ser^{789}) by ERK1/2 reduces its inhibitory effect on the myosin ATPase activity. For example, in the VSMCs of pulmonary artery, ERK1/2 is activated by growth factors such as PDGF and, via phosphorylation of CaD, modulates che-

motactic migration.[45] PDGF also increases the intracellular calcium concentration and activates ERK1/2 in a Ca^{2+}-dependent manner in VSMCs.[46] In the present study, the phosphorylation of ERK1/2 was reduced by overexpression of *l*-CaD via a negative-feedback mechanism on the ERK/*l*-CaD pathway. These results suggest the presence of crosstalk between PDGF and CaD. We consider that this reduction of ERK1/2 phosphorylation may also contribute to suppression of cell proliferation and migration in vitro, and also the neointimal formation of rat balloon-injured arteries.

h-CaD is present in differentiated SMCs, whereas *l*-CaD is present in nonmuscle cells and in dedifferentiated SMCs, and both isoforms are expressed from a single gene via alternative RNA splicing.[22,23] The protein switches from the *l*-form to the *h*-form during SMC differentiation, and from the *h*-form to the *l*-form during dedifferentiation.[47] Therefore, *h*- and *l*-CaD are considered markers of SMC differentiation and dedifferentiation, respectively. Although the VSMC phenotype-dependent splicing of CaD is still undergoing investigation, the CaD promoter activity and expression of *h*-CaD in differentiated VSMCs is higher than *l*-CaD in dedifferentiated VSMCs.[47] From the findings of *l*-CaD overexpression in VSMCs and the association of CaD-isoform interconversion with VSMC phenotypic modulation, we speculate that *l*-CaD may be able to inhibit cell proliferation. *l*-CaD may be induced to inhibit excessive proliferation of VSMCs during the usual process of vascular wound healing after balloon injury. In contrast, failure to exert this suppression may contribute to excessive neointima formation during restenosis. By overexpressing *l*-CaD, we were able to restore the inhibitory balance and suppress proliferative responses to mechanical injury. Taken together, these findings suggest that the abundance of *l*-CaD expression in VSMCs may indicate not only the degree of dedifferentiation but also the degree of mechanical stress in response to the vascular injury. Further investigations are required into this suppressive role of *l*-CaD and the underlying regulatory mechanism responsible for control of VSMC proliferation.

Regarding the clinical application of *l*-CaD in preventing restenosis, the greater effect of *l*-CaD overexpression on inhibiting the proliferation of ECs (compared with VSMCs) will result in denuded ECs after a routine stenting procedure, and we confirmed no difference in the rate of re-endothelialization in this balloon-injured carotid artery model. Furthermore, *l*-CaD does not result in prolonged endothelial damage such as clot formation and inhibition of re-endothelialization, as reported for paclitaxel and other immunosuppressants, but rather is a contractile protein that is ubiquitously expressed in many tissues under strict genomic control of the RNA splicing system. Therefore, a transient overexpression of *l*-CaD within mechanically injured arteries would suppress proliferating cells such as VSMCs and fibroblasts without serious inflammation or other excessive immune responses in regenerated ECs. However, applying a cytostatic strategy with *l*-CaD requires an efficient local gene delivery system to be developed.

In conclusion, we have demonstrated that overexpression of *l*-CaD suppresses the proliferation and migration of VSMCs via the cytoskeletal tension-FAK-ERK1/2 axis, and inhibits neointimal formation after balloon injury in rat carotid arteries. This finding may open new avenues for therapeutic intervention in patients with vascular restenosis.

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Disclosures

None.

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