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The Transfection of Caldesmon DNA into Primary Cultured Rat Aortic Vascular Smooth Muscle

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Caldesmon (CaD), one of microfilament-associated proteins, plays a key role in microfilament assembly in mitosis. We have investigated the effects of overexpression of the high molecular weight isoform of CaD (h-CaD) on the physiology of vascular smooth muscle cells (VSMCs). Rat aortic VSMCs were stably transfected with plasmids carrying a full length human h-CaD cDNA under control of cytomegalovirus promoter. The majority of the overexpressed h-CaD appears to be localized predominantly on cytoskeleton structures as determined by detergent lysis. The overexpression of h-CaD, however, does not decrease the level of endogenous low molecular weight isoform of CaD. h-CaD overexpressing VSMCs (h-CaD/VSMCs) show a decreased growth rate than that of vector-only transfected cells when determined by [^{3}H]thymidine uptake and cell counting after fetal bovine serum (FBS) stimulation. h-CaD/VSMCs were smaller than vector-transfected cells by 18% in cell diameter. These data suggest that overexpression of h-CaD can inhibit the poliferation and the cell volume of VSMCs stimulated by growth factors and that the gene therapy with h-CaD may be helpful to prevent the conditions associated with hypertrophy and/or hyperplasia of VSMCs after arterial injuries.

Key Words: Caldesmon, Overexpression, Vascular smooth muscle cell, Growth inhibition, Cell volume decrease

INTRODUCTION

Caldesmon (CaD) is a protein found on actin-based filaments of cells and it can bind to actin, myosin, tropomyosin, and Ca^{2+}/Calmodulin. CaD can enhance tropomyosin binding to actin and inhibit ATPase activity of actomyosin, These characteristics are reversibly regulated by Ca^{2+}/Calmodulin and phosphorylation state of CaD. Alternative splicing of one gene produces two isoforms of human CaD; a high molecular weight form, h-CaD, and a low molecular weight form, l-CaD, with an apparent molecular weight 130~150 kDa and 70~80 kDa on SDS-PAGE, respectively (reviewed in Huber, 1997; Hayashi et al, 1991).

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Both isoforms of CaD perform different roles in vivo. This may be reflected by the distinct cellular distribution of these isoform classes. l-CaD is found in non-muscle and in dedifferentiated smooth muscle cells and is regarded to be a regulatory factor in the microfilament network and is thus involved in the assembly and stabilization of microfilaments. l-CaD is part of stress fiber and may thus be involved in changes of cell shape. As h-CaD is exclusively found in adult and fully differentiated smooth muscle cells, h-CaD together with tropomyosin is thought to be a mediating factor for inhibition of smooth muscle contraction (reviewed in Yamashiro et al, 1994).

Vascular smooth muscle cells (VSMCs) undergo phenotypic modulation, from differentiated contractile state to dedifferentiated proliferative state, in some disease conditions associated with arterial injuries such as atherosclerosis and hypertension and even in conventional *in vitro* culture conditions where fetal bovine serum is usually supplemented (Sobue et al,

1998). During this phenotypic modulation, VSMCs change their morphology, cell function and biochemical characteristics. Recent studies have revealed that CaD is one of VSMC-specific genes which are regulated at the levels of transcription and splicing in a phenotype-dependent manner and that h-CaD is converted into l-CaD in this process (Kashiwada, 1997).

In this article, the effects of h-CaD overexpression were examined in dedifferentiated VSMCs on the physiology of these cells and we demonstrate that h-CaD overexpression in VSMCs can inhibit proliferation of cells and decrease cell diameter leading to reduced cell volume.

METHODS

Cell culture

Rat aortic vascular smooth muscle cells (VSMCs) were isolated by enzymatic dispersion with a slight modification of the method described by Chamley et al (1977). The thoracic aorta from Sprague-Dawley rats (6 to 8 weeks old, Chung Ang Experimental Animals, Korea) were removed and transferred on ice in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4) containing 100 μg/ml penicillin/streptomycin. The aorta was freed from connective tissue; was transferred into a glass dish containing 3 ml of a dissociation enzyme mixture containing DMEM with 400 IU/ml collagenase type I, 0.5 mg/ml elastase, and 0.5 mg/ml soybean trypsin inhibitor; and was incubated for 30 minutes at 37°C. Then the aorta was transferred again into PBS, and the adventitia was stripped off with forceps. The aorta was minced, and the minced media was transferred into a plastic tube containing 6 ml of the dissociation enzyme mixture. The suspension was incubated for 2 hours at 37°C under constant agitation and then centrifuged ($400\times g$ for 10 minutes). The pellet was resuspended in DMEM with 10% fetal bovine serum (FBS) and plated in a 100 mm culture dish. The resulting VSMCs were cultured over several passages according to Chamley-Campbell et al (1981). The purity of VSMCs was confirmed by immunocytochemical localization of smooth muscle-specific α-actin with fluorescein isothiocyanate-conjugated antibody (data not shown). Experiments were performed with VSMCs in passage 6 to 9.

Expression vector construction and DNA transfection

pSKHCDM5, a plasmid harboring cDNA for human high molecular weight isoform of caldesmon (h-CaD, Humphrey et al, 1992; genebank accession M83216), was kindly provided by Dr. J. Bryan (Baylor College of Medicine, Houston, TX, U.S.A.). Xba I fragment (−241 to 2781 nucleotides) spanning the whole open reading frame (241 to 2622 nucleotides) for h-CaD was subcloned into the Xba I site of pcDNA3 (Invitrogen) downstream of the cytomegalovirus promoter and the orientation of insert was confirmed with restriction enzyme digestion. ph-CaD (Fig. 1A), the resulting expression vector for human h-CaD, was propagated in *E. coli* DH5α. ph-CaD plasmid DNA for VSMC transfection was prepared by alkaline lysis and acid phenol treatment. VSMCs were transfected with ph-CaD DNA and SuperFect (Quiagen) according to the manufacturer's recommendation. ph-CaD transfected VSMCs were selected with 200 μg/ml Geneticin for three weeks. The selection concentration of Geneticin was determined empirically as such 90% of VSMCs seeded at a density of 1.2×10^5 cells/100 mm dish were killed in 6 days. As a control, vector-only transfected VSMCs (pcDNA3/VSMCs) were also selected.

Immunoblot

To study the distribution of h-CaD in cell fractions, VSMCs on 100 mm culture dish were rinsed twice with ice-cold PBS, then treated with 1.5 ml of solubilization solution (50 mM NaCl, 25 mM $MgCl_2$, 2 mM EGTA, 0.1% Triton X-100, 0.1 mM PMSF and 2 μg/ml chymostatin/leupeptin). The Triton X-100 solubilized samples were centrifugated at $15{,}000\times g$ for 30 minutes at 4°C. The supernatant and the pellet were separately collected after centrifugation. The pellet was sonicated in 1X SDS-PAGE sample buffer without 1% 2-mercaptoethanol and 0.1% bromophenol blue (15% glycerol, 125 mM Tris pH 6.8, 5 mM EDTA, 2% SDS). The protein concentrations of the pellet suspension and the supernatant was determined using Bradford protein assay (Bio-Rad) with BSA as a standard. Equal amounts of protein were loaded on 7.5% polyacrylamide gels, transferred to PVDF membrane (Bio-Rad) and probed with anti-CaD monoclonal antibody (Sigma, C6542) which can localize h-CaD and l-CaD in cultured human smooth muscle cells (Durand-Arczynska et al, 1993). The

immunoreactive bands were visualized with the ECL system (Amersham).

[^{3}H]thymidine uptake assay

VSMCs were seeded in 24-well plates and cultivated till 70% confluency was reached. The medium is replaced by serum-free quiescent medium consisting of DMEM and Ham's F-10 medium (1 : 1). After 24 hours, 10% FBS was added to the VSMCs. After another 44 hours, [^{3}H]thymidine was added to the medium at a final concentration of 1 μCi/ml. The VSMCs were labelled for another 4 hours and the labelling was terminated by aspirating the medium and subjecting the cells to sequential washes on ice with PBS containing 10% trichloroacetic acid, and ethanol/ether (2 : 1, vol/vol). Acid-insoluble [^{3}H]thymidine was extracted into 250 μl of 0.5 M NaOH per well, and 0.1 ml of this solution was mixed with 5 ml scintillation cocktail (Packard, Ultima Gold) and quantified with a liquid scintillation counter (model LS 3801, Beckman). Fifty microliters of the residual solution was prepared for the determination of protein with Bradford protein assay (Bio-Rad).

Cell count

In 24-well plates, VSMCs were plated at a density of 1×10^4 cells/well in serum-free quiescent medium. After 24 hours, cell growth is stimulated by adding 10% FBS to the medium. For counting of cell number at determined time interval, VSMCs were washed with ice-cold PBS three times, fixed with AFA solution (20 : 2 : 1 mixture of ethanol, acetic acid, and 40% formaldehyde) for 10 minutes and stained with Mayer's hematoxylin solution (Sigma, MHS-128) for 30 minutes. With an inverted microscope, the number of cells in each well was counted in randomly chosen 4 fields in a well under a low power field ($\times$ 100).

FACS analysis of cell diameter

VSMCs were harvested by trypsinization and resuspended in FACS buffer (PBS with 0.1% BSA). The cell suspension was centrifugated and the resulting pellet was resuspended again in fixing solution (FACS buffer with 4% paraformaldehyde) at a density of 1×10^5 cells/ml. FSC, a cell-diameter proportional parameter, was measured from 1×10^4 VSMCs and analyzed with Calibur-S FACScan (Becton Dickinson, U.S.A.).

Statistical analysis

All data are represented as means $\pm$ standard errors of means. Differences between groups were determined by Student's *t*-test (Fig. 3 and Fig. 5), or by analysis of variance (ANOVA) and Duncan's multiple comparison (Fig. 4); were considered significant when *p*-values were of less than 0.05.

RESULT

Overexpressed human h-CaD is predominantly associated with cytoskeleton

The cellular localization of overexpressed human h-CaD was determined by cell fractionation. Using 0.1% Triton X-100 as a detergent described in Materials and Methods, cells were separated into a soluble protein fraction and a detergent insoluble cytoskeleton fraction. The molecular weight of immunoreactive bands was estimated as 120 kDa and 80 kDa.

Before completing the G418 selection, in the cytoskeleton fraction of ph-CaD/VSMCs, there is a immunoreactive band at 80 kDa, which was thought to be l-CaD that is endogenously expressed in dedifferentiated VSMCs (Fig. 1B). l-CaD immunoreactivity is possibly due to a cross reaction to monoclonal antibody between rat and human CaD. As 90% of untransfected VSMCs were expected to be killed under G418 selection, less than 0.1% of total number of VSMCs are expected to be untransfected at the end of three passages selection period. In a cytoskeleton fraction, the major band was at 80 KDa in VSMCs before G418 selection. After G418 selection, however, the intensity of 120 KDa band increased and this band was thought to be the overexpressed h-CaD. In a detergent soluble fraction, there was no 80 KDa band before the selection. When the selection was finished, however, there appeared both 120 KDa and 80 KDa band.

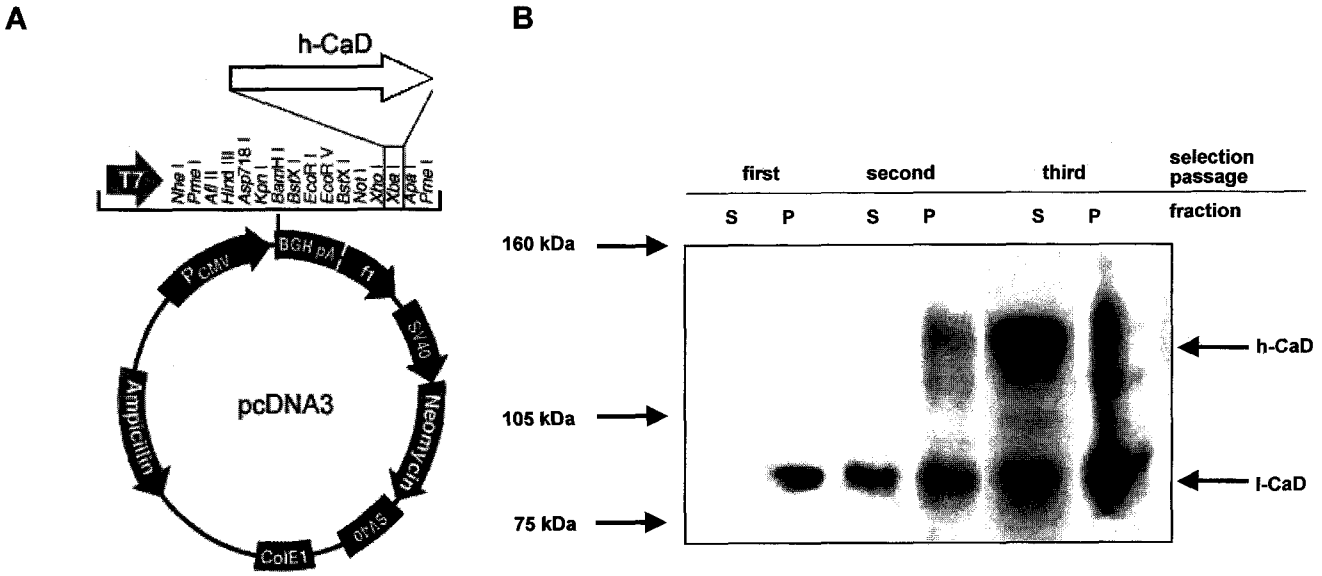

Fig. 1. The expression vector map and the expression pattern of h-CaD during selection period. (A) h-CaD insert is subcloned into pcDNA3. pCMV is cytomegalovirus promoter which controls the expression of h-CaD in VSMCs. (B) There are changes of the localization of h-CaD and l-CaD in a detergent soluble fraction (S) and a detergent insoluble cytoskeleton pellet (P) during three passages of G418 selection period, when ph-CaD/VSMCs were prepared after every passage (first, second and third).

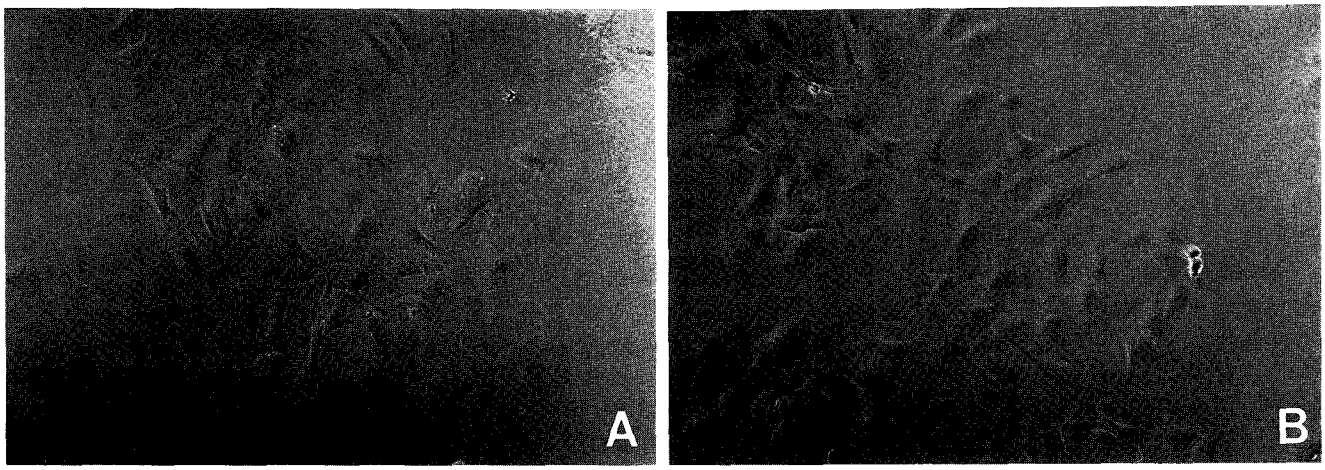

Fig. 2. The morphology of ph-CaD/VSMCs and pcDNA3/ VSMCs. pcDNA3/VSMCs (A) have plump cytoplasmic area and ph-CaD/VSMCs (B) are more enlongated and embossed than pcDNA3/VSMCs.

The morphological alterations in VSMCs overexpressing human h-CaD

pcDNA3/VSMCs spreaded widely on the culture dish and the margin of a single cell is polygonal or round. They had a prominent nuclei, which made the cells look centrally embossed (Fig. 2). The ph-CaD/VSMCs looked more enlongated and embossed than pcDNA3/VSMCs. In FACS analysis, the diameter of ph-CaD/VSMCs were smaller than that of pcDNA3/VSMCs by 18%. This decrease in diameter corresponds to 33% decrease in surface area and 45% decrease in cell volume (Fig. 3).

Overexpression of human h-CaD inhibits the proliferation of fetal bovine serum (FBS) stimulated VSMCs

Although the dissociation of CaD from microfi-

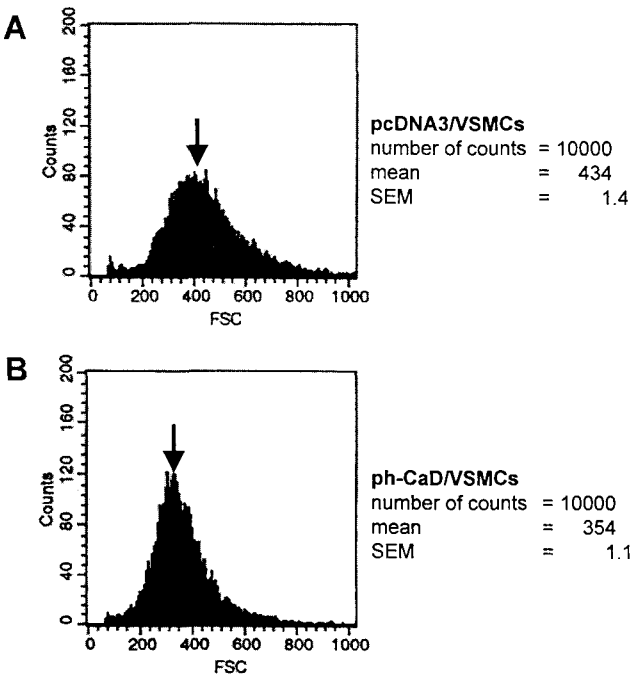

Fig. 3. FACS analysis for cell diameter. pcDNA3/VSMCs (A) and ph-CaD/VSMCs (B) were analyzed for FSC, which is proportional to the diameter of each cell. Vertical arrows in each panel denote the location of mean FSC, which is different significantly from each other. ($p < 0.05$).

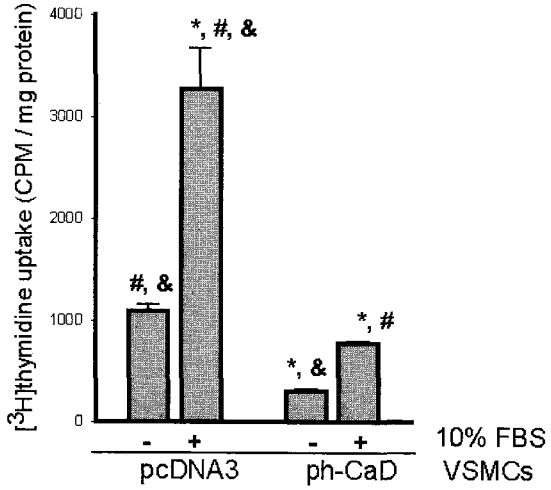

Fig. 4. Effects of h-CaD overexpression on DNA synthesis. [^{3}H]thymidine uptake was measured in pcDNA3/VSMCs and ph-CaD/VSMCs with (+) or wihtout (−) the growth stimulation of 10% FBS. Each bar represents mean and standard errors of mean in each group (n=4). The mean of each group is significantly different from that of pcDNA3/VSMCs without FBS stimulation (*), ph-CaD/VSMCs without FBS stimulation (#), or ph-CaD/VSMCs with FBS stimulation (&).

laments as a consequence of mitosis-specific phosphorylation is a prerequisite to cell division, it is not yet determined in VSMCs whether h-CaD could participate in the regulation of cell growth, since the main function of h-CaD in VSMCs is thought to be regulation of contractile elements. The proliferation of VSMCs after growth stimulation was determined by [^{3}H]thymidine uptake assay (Fig. 4). Basal [^{3}H]thymidine uptake was significantly lower in ph-CaD/VSMCs than pcDNA3/VSMCs by 3.6 fold. Stimulation with FBS increased the [^{3}H]thymidine uptake by 2.6 fold in ph-CaD/VSMCs and 3.0 fold in pcDNA3/VSMCs resulting that FBS-stimulated [^{3}H]-thymidine uptake of ph-CaD/VSMCs is less than that of pcDNA3/VSMCs by 4.3 fold. Although the number of FBS-starved VSMCs also decreased, the number of ph-CaD/VSMCs was not significantly less than that of pcDNA3/VSMCs. After two days of FBS stimulation, there is a 4.2 fold increase in the number of ph-CaD/VSMCs and pcDNA3/VSMCs showed 6.5 fold increased cell number in comparison to those of FBS-starved cells. The number of ph-CaD/VSMCs are 1.6 fold less than that of pcDNA3/VSMCs after FBS stimulation.

DISCUSSION

When h-CaD is overexpressed in VSMCs, preferential association site of h-CaD seems to be cytoskeleton since the overexpressed h-CaD began to appear in a Triton X-100 insoluble pellet fraction. When the stable transfectant of h-CaD were selected with G418, not only h-CaD but also l-CaD appeared in a soluble fraction. As the domains with a strong actin-, tropomyosin-, myosin- and calmodulin-binding activity reside in the area which is overlapped between l-CaD and h-CaD (Huber, 1997), l-CaD and h-CaD are expected to show competetion to the same intracellular binding sites. When h-CaD expression is far greater than the endogenous l-CaD expression, h-CaD might replace l-CaD on the same binding (or association) sites in cytoskeleton and l-CaD should remain unbound from cytoskeleton structures to appear in a detergent soluble fraction.

The VSMCs undergo phenotypic changes from a differentiated contractile to dedifferentiated proli-

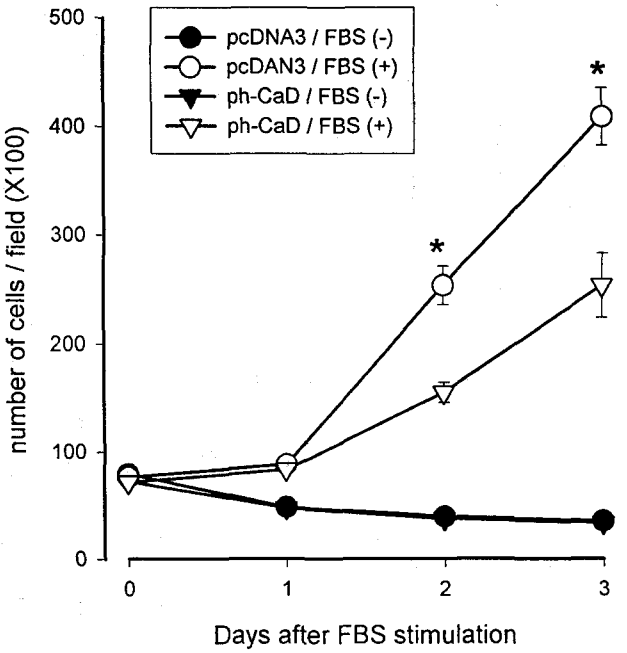

Fig. 5. Effects of h-CaD overexpression on proliferation of VSMCs. For three days (horizontal axis) after FBS stimulation, cell number was counted for ph-CaD/VSMCs and pcDNA3/VSMCs with or without FBS stimulation. Each point represents mean and standard errors of mean of cell count (n=6). Asterisk (*) indicates that the mean of cell count is significantly different between ph-CaD/VSMCs and pcDNA3/VSMCs when VSMCs are stimulated with 10% FBS.

ferative state under conventional culture conditions, in which 10% FBS is supplied. During dedifferentiation, the morphology of VSMCs shows a change, from a long spindle-like to a fibroblast-like shape. During this process, h-CaD, which is specific for the differentiated phenotype of VSMCs, is converted to l-CaD (Kashiwada et al, 1997). As the immunoblot results suggested that there had been already a isoform change in CaD expression before transfection with ph-CaD and pcDNA3 (Fig. 1B), we wondered whether overexpression of h-CaD again in these VSMCs can reverse the phenotypic changes. There are alterations in the morphology of ph-CaD/VSMCs. Although the diameter of these cells decreased in FACS analysis, these cells were more enlongated and looked more embossed. They did not recover their characteristic spindle-like morphology of primary cultured VSMCs, which is known as hill-and-valley shape. These data suggest that the isoform change itself might not be the only requirement of differentiation, although a change of CaD isoforms from l-CaD to h-CaD is closely linked to VSMC differentiation. On the other hand, this might be due to a continuous expression of l-CaD in ph-CaD/VSMCs. Immunoblot results have shown that h-CaD overexpression itself could not suppress the synthesis of l-CaD in VSMCs and that l-CaD band did not disappear from the cytoskeleton fraction until the end of G418 selection.

When h-CaD is overexpressed in mouse fibroblast cell line (L cell), these cells acquire a distinct phenotype characterized by an altered morphology, including an increased number of processes and larger area due to enhanced cell spreading (Surgucheva & Bryan, 1995). Warren et al (1996), however, found that CaD39 (carboxyl-terminal fragment of CaD) expressing CHO cells exhibited enhanced spreading immediately after attachment to culture dish and that these cells were more elongate and encompassed less area than non-expressing cells during migration in a wound-healing assay, suggesting some of the effects we observe with overexpressed h-CaD might be dependent on the cell-type and/or the domain of CaD that is overexpressed.

It has been revealed that CaD inhibits the tropomyosin-stimulated, actin-activated ATPase of myosin in reconstituted systems and that this inhibition is attenuated by Ca^{2+}/calmodulin, which reverses actin binding by CaD. Actin-bound CaD can be phosphorylated by p34cdc2 kinase in a mitosis specific manner, resulting in dissociation from F-actin (Mak et al, 1991). The dissociation of CaD may release the inhibition of actomyosin ATPase and may lead to contraction of actin-myosin system in mitotic cells, causing rounding-up of cell shape when cells enter prophase. These suggest that the inhibition of most of CaD's functions may be a required step in the massive reorganization of microfilaments seen during mitosis (Yamashiro et al, 1991; Yamakita et al, 1992).

Overexpression of CaD might be lethal since it can cause disorganization of microfilaments including disassembly of stress fibers, resulting in cell rounding and death. However, microscopic examination of ph-CaD/VSMCs did not find any rounded cells. These results suggest that the lethal expression of h-CaD can be ruled out at least in VSMCs.

In this experiment, ph-CaD/VSMCs show a less proliferation than pcDNA3/VSMCs cells after FBS stimulation, although there is a discrepancy between

the decrease fold of [^{3}H]thymidine uptake and cell number. In addition to the slowing of cell growth, the cell volume is also decreased. Hyperplasia of VSMCs is one of key changes in several pathologic conditions, such as hypertension, restenosis after baloon angioplasty and atherosclerosis, where arterial injuries are implicated. Although there are many pharmacological agents which can inhibit the proliferation of VSMCs, there are a few agents that can inhibit the hypertrophy of VSMCs and there is no agent which do not cause any systemic unwanted effects that many patients suffer from.

Our data suggest that h-CaD overexpression might prevent the arterial narrowing and thickening by reducing the number and the volume of growth stimulated VSMCs and in conclusion, that gene therapy with h-CaD may permit the development of new strategies to inhibit the proliferative response of VSMCs to arterial injuries.

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