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Stretch affects phenotype and proliferation of vascular smooth muscle cells

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Abstract

The exertion of periodic dynamic strain on the arterial wall is hypothesized to be relevant to smooth muscle cell morphology and function. This study has investigated the effect of cyclic mechanical stretching on rabbit aortic smooth muscle cell proliferation and expression of contractile phenotype protein markers. Cells were cultured on flexible-bottomed dishes and cyclic stretch was applied (frequency 30 cycles/min, 15% elongation) using a Flexercell Strain unit. Cyclic stretch potentiated smooth muscle cell proliferation in serum-activated cultures but not in cultures maintained in 0.5% fetal calf serum. Stretching induced a serum-independent increase of h-caldesmon expression and this effect was reversible following termination of mechanical stimulation. Strain was without effect on smooth muscle myosin or calponin expression. In cells grown on laminin stretch-induced h-caldesmon expression was more prominent than in cells cultured on collagen types I and IV, poly-L-lysine and gelatin. These data suggest that cyclic mechanical stimulation possesses dual effect on vascular smooth muscle cell phenotype characteristics since it: 1) potentiates proliferation, an attribute of a dedifferentiated phenotype; and 2) increases expression of h-caldesmon considered a marker of a differentiated smooth muscle cell state. (Mol Cell Biochem **144:** 131–139, 1995)

Key words: cyclic stretching, VSMC, proliferation, phenotype markers, caldesmon

Introduction

Smooth muscle cells within the arterial wall are normally subjected to mechanical deformation in the form of cyclic stretch (CS) produced by a pulse wave. Such periodic dynamic forces are physiologically relevant to the regulation of vascular smooth muscle cell (VSMC) morphology and function. Physiological studies have demonstrated that stretching of aortic strips causes rapid and transient increases in intracellular Ca^{2+} and phosphorylation of myosin regulatory light chains and these effects are followed by the myogenic contraction of vascular smooth muscle [1]. In atherosclerotic lesions and intimal thickenings that occur after balloon catherterization, as well as in artery-graft joints both an activation of proliferation [2] and phenotypic modulation of VSMC [3] takes place. The latter includes the reduction of volume fraction of myofilaments [3, 4] and decreased expression of smooth muscle actin [5, 6], myosin [7] and caldesmon [8]. Factors demonstrated to affect VSMC phenotype in these pathologies include serum- and platelet-derived mitogens, and the interaction of VSMC with other cell types. In addition, abovementioned vascular alterations are accompanied by a reduced mechanical deformation of the vessel wall due to a decreased elasticity of damaged areas. Hence, the lack of appropriate mechanical stimulation should also be considered as a potential factor influencing VSMC phenotype.

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Long term effects of CS have already been demonstrated in a variety of cell types. Mechanical stimulation of embryonic cardiomyocytes induces a transient increase in expression of immediate response gene c-fos [9], and this is followed by expression of new actin isoform, increased production of atrial natriuretic peptide and increased total protein synthesis [10, 11]. Mechanical stimulation of endothelial cells potentiates an activation of prostacyclin release [12] and stimulates a production of tissue plasminogen activator by endothelial cells [13]. CS has been shown to stimulate the growth of fibroblasts and cultured endothelial cells [9, 14]. Cultured vascular smooth muscle cells respond to long-term CS with an increased production of extracellular matrix [15] and synthesis of PDGF [16]. While such effects indicate that besides functional features CS may regulate cellular events pertinent to VSMC phenotypic modulation, the relationship between mechanical stimulation and VSMC phenotype has not yet been studied.

In order to assess the influence of mechanical stimulation on VSMC phenotype we have utilized a culture model in which VSMC can be subjected to periodic cyclic strain. The expression of the contractile phenotype marker proteins *h-caldesmon and calponin as well as smooth muscle myosin heavy chains (SMM) was analysed. Calponin (CN) and a smooth muscle variant of caldesmon (h-CaD) are abundant actin-binding proteins which regulate smooth muscle contraction in a Ca^{2+}-dependent manner [18]. The expression of h-CaD and CN as well as SMM drastically decreases following VSMC phenotypic modulation from a contractile to a synthetic form *in situ* and in culture [8, 19, 20] and correlates with a loss of the ability of cultured VSMC to contract [20]. The influence of CS on VSMC proliferation was also studied. We furthermore report on how stretch-mediated effects on VSMC phenotype and proliferation are influenced by serum factors and underlying matrix substratum.

Materials and methods

Reagents

Elastase (type IV from bovine pancreas), Collagenase (type IV from Clostridium histolyticum), and soybean trypsin inhibitor were purchased from Boehringer Mannheim AG (Switzerland). Minimal essential medium (MEM), phosphate buffered saline (PBS), L-glutamine, penicillin, and streptomycin were from GIBCO (Basel, Switzerland). Tissue culture plasticware was purchased from Costar (Tecnomara AG, Switzerland). Nitrocellulose (0.45 μm) was from Bio-Rad (Switzerland). All other reagents were of tissue culture grade or analytical grade.

Cell culture and cell enumeration

VSMC from the media of 9- to 12-week-old rabbit aortae were isolated enzymatically according to Chamley-Campbell *et al.* [21] and cultured in petri dishes in MEM supplemented with 10% fetal calf serum (FCS), 20 mM L-glutamine, 10 mM TES-NaOH/HEPES-NaOH (pH 7.3), 100 U/ml penicillin, 100 μg/ml streptomycin in a humidified atmosphere (37°C; 5% CO_2). Culture medium was replaced every 2 days. Cells were subcultured using 0.05% trypsin for dissociation and cultures between passages 2–5 were used in this study. Freshly isolated VSMC were phenotypically characterized using polyclonal antibodies against smooth muscle myosin and caldesmon. For cell enumeration, cells were detached from culture plates using 0.05% trypsin/0.5 mM EGTA in Hank's buffer and then suspended in 0.9% NaCl and counted using an electronic counter (Coulter, Instrumenten-Gesellschaft AG, Basel, Switzerland). Viability of attached cells was 90% +/–5% as revealed by Trypan blue dye exclusion test and did not change significantly under all experimental conditions used.

CS experiments and optimization of culture conditions

VSMC were seeded onto Flex culture plates coated with either type-I, or type-IV collagens, or laminin (Flexcell Corp., McKeesport, USA) in MEM supplemented with 10% FCS at an initial density 2–8 × 10^4 cells/cm^2. When necessary, untreated Flex plates were hydrophylized and coated with poly-L-lysine or gelatin as previously described [22]. After 48 h incubation to allow cell attachment and spreading, culture medium was replaced by MEM supplemented as above but containing 0.2% bovine serum albumin instead of FCS. Cultures were maintained for 24 h under serum-free conditions to obtain quiescent non-dividing cells, and then fresh medium containing selected concentrations (0.5–10%) of FCS was added. Flex culture plates were positioned onto the gasketed baseplate of a computerised Flexercell strain unit, placed in the incubator and then subjected to cyclic mechanical stretch (30 cycles/min; 0.5 sec stretching, 1.5 sec relaxation; 15% elongation at the periphery of the culture plate bottom) for up to 8 days. Plating efficiency of VSMC seeded onto Flex plates was measured after 48 h of culture and comprised 80% +/–7% of total cell amount introduced in the well. Number of attached VSMC after 24 h of CS comprised 75% +/–5% of cells present in the well prior to stretching session and did not vary significantly at all culture conditions studied. Parallel series of Flex culture plates not subjected to cyclic

*Molecular weights of caldesmon variants deduced on the basis of their sequences are in the range of 87–89 and 59–60 kDa, respectively, and are different from those determined by electrophoretic methods [17]. In this study, to distinguish between caldesmon variants we used the technique of immunoblotting, and therefore used the terms h-CaD for high molecular weight caldesmon and l-CaD for low molecular weight isoform.

stretching served as controls. VSMC cultured in serum-free medium (MEM containing 0.2% BSA, 5 ng/ml selenite, 5 μg/ml transferrin and 5 μg/ml insulin) did not survive well under prolonged serum-free conditions, and about 80% of VSMC detached from both stretched and control plates after the fourth day of culture (data not shown). Pilot studies indicated that a minimal FCS concentration of 0.5% was necessary to maintain VSMC attachment (data not shown). In some experiments, non-stretched culture plates were mounted onto a 'Rocky-shaker' to control for effects arising from stirring of the culture medium, and these plates were nutated at the same frequency as CS. At the end of culture protocols, medium was aspirated and cultures rinsed with ice-cold PBS before cell enumeration and the preparation of samples for electrophoresis and immunoblot analysis of marker protein expression.

Marker protein expression: antibodies and immunoblotting techniques

Rabbit polyclonal affinity-purified antibodies against chicken gizzard caldesmon, chicken gizzard calponin, and chicken gizzard smooth muscle myosin have been characterized previously [23–25]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [26] using 9% gels for detection of calponin and 6% gels for detection of caldesmon and smooth muscle myosin. Proteins were electrophoretically transferred from polyacrylamide gels onto nitrocellulose according to Towbin *et al.* [27]. Quantitative immunoblotting on nitrocellulose membranes was performed as previously described [28]. Polyclonal antibodies to caldesmon, smooth muscle myosin and calponin were used at a concentration of 4 μg/ml. [^{125}I]-labelled secondary antibodies at a final concentration of 0.1 μCi/ml (Amersham, Zurich, Switzerland) were used for the immunoreactive quantitation of marker proteins. Scanning densitometry of Coomassie R-250-stained gels was performed using a laser scanner (LKB Bromma, Sweden) to determine total cell protein. The amount of a given smooth muscle marker protein is expressed as that amount of radioactivity incorporated into the protein band normalized with respect to the total cell protein. Samples for immunoquantitative and scanning densitometric comparison were run on the same gel and processed simultaneously, and both methods were linear within the range of protein loadings used. Immunoblot analysis revealed the presence of all tested marker proteins in both primary and subcultured rabbit VSMC although the levels of smCaD, CN and SMM in passaged cells were markedly lower (data not shown).

Statistical analysis

Data were expressed as the means ± SD. A statistical analysis was carried out by unpaired Student's *t* test and a value of $p < 0.05$ was considered to be statistically significant.

Results

Effects of cyclic stretch on VSMC attachment and proliferation

We assessed the influence of CS on cell attachment by determining the proportions of attached and detached VSMC in cultures either subjected to CS or maintained under stationary conditions. VSMC were seeded onto stretch plates in culture medium containing 10% FCS. After 48 h culture medium was changed for additional 48 h on that containing 0.2% BSA to obtain quiescent population. After synchronization of VSMC medium containing 0.5, 2, 5, or 10% FCS was added and cells were cultured for 4 days with or without CS. On day 2 and 4 culture medium was collected from the wells and detached cells were enumerated. Two measurements were combined to give the final absolute value for cell detachment. Cell attachment was measured on day 4 following trypsinization and cell enumeration. For both stretched and control cultures the absolute numbers of detached cells increased with increasing concentrations of FCS and were in good correlation with the serum-dependent proliferative activity of VSMC (Table 1). The proportions (as % of total cell numbers) of detached and attached VSMC were not different between control and stretched cultures (Table 1) indicating that CS does not influence the ability of cells to attach to the membranes. However, CS evidently influences the proliferative activity of VSMC since absolute cell numbers (detached or attached) were consistently higher in stretched cultures. (Table 1).

For VSMC cultured in the presence of 0.5% FCS, the onset of proliferation was detectable on the fourth day in culture both either without or with CS (Fig. 1). Growth promoting effects of CS were absent during the non-proliferating (quiescence) period.

To discriminate effects of stretching and those arising from the mere motion of culture medium in stretched plates VSMC were cultured under stationary, CS or nutatory conditions. VSMC proliferation examined in the presence of 2% FCS was not different between stationary and nutated cultures (Fig. 2). Thus the effects of CS on serum-stimulated proliferation reflect a true stretch-related phenomenon, rather than the outcome of improved homeostatic culture conditions.

CS-mediated expression of smooth muscle phenotype protein markers

The effects of CS on VSMC phenotype were studied by following the expression of protein markers associated with the contractile VSMC phenotype, namely h-caldesmon, calponin and smooth muscle myosin [20, 29]. Passaged VSMC used for this study expressed low amounts of h-CaD (about 10%

Table 1. Attachment efficiency of cultured VSMC under stationary conditions and during cyclic stretching

Culture conditions	Detached cells ($\times 10^{-4}$)	Attached cells ($\times 10^{-4}$)	Total cell number ($\times 10^{-4}$)	Detached cells (%)	Attached cells (%)
MEM + 0.5% FCS					
control	3.4 ± 0.2	3.5 ± 0.3	6.9	48.8	51.2
stretch	3.3 ± 0.2	4.0 ± 0.2*	7.3	42.3	57.7
MEM + 2% FCS					
control	4.1 ± 0.3	4.8 ± 0.3	8.9	46.4	53.6
stretch	3.9 ± 0.2	6.6 ± 0.3*	10.5	37.1	62.9
MEM + 5% FCS					
control	6.1 ± 0.4	6.2 ± 0.4	12.3	49.5	50.5
stretch	8.5 ± 0.2	8.5 ± 0.3*	17.0	50.0	50.0
MEM + 10% FCS					
control	6.3 ± 0.3	7.4 ± 0.3	13.7	45.8	54.2
stretch	8.8 ± 0.4	11.2 ± 0.4*	20.0	44.0	56.0

VSMC seeded on collagen I-coated Flex dishes (3.5×10^4 cells/well) were cultured for 4 days in the presence of the indicated concentrations of FCS and without or with application of CS. Numbers for detached cells represent the sum of cell numbers determined on days 2 and 4. Attached cells were determined on day 4. These numbers are given as mean ± SD and were obtained from triplicate determinations in 4 separate experiments. All methodological details are given in Materials and methods. *$p < 0.05$ vs control.

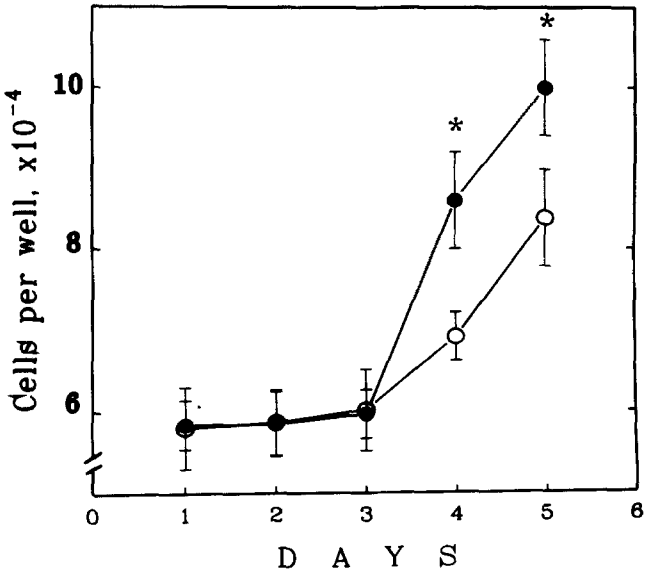

Fig. 1. Effects of CS on growth of VSMC in the presence of 0.5% serum. VSMC seeded on collagen I-coated Flex plates (6×10^4 cells/well) were cultured for the indicated periods of time in stationary conditioned (open symbols) or were subjected to CS (closed symbols) and cell numbers determined. Values given represent mean ± SD of data obtained in 3 separate experiments in each of which triplicate determinations were made. *$p < 0.05$.

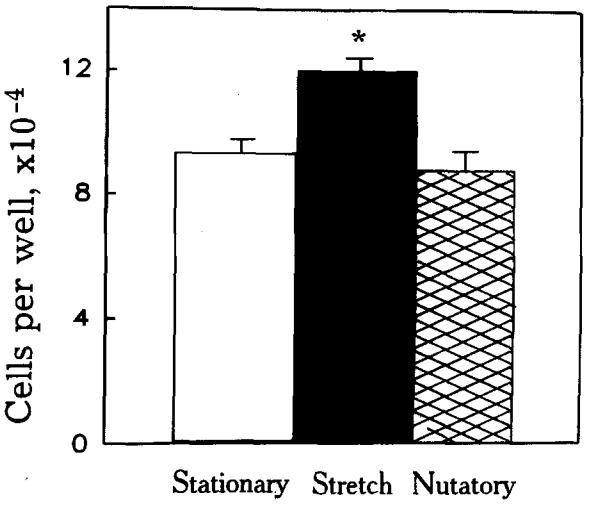

Fig. 2. Growth of VSMC under stationary, nutatory and stretch culture conditions. VSMC on collagen I-coated Flex dishes were cultured for 4 days in the presence of 2% FCS under either stationary (open bars), stretch (closed bars) or nutatory (hatched bars) conditions as described in Materials and methods. Cell enumeration was performed after 4 days. Values given represent mean ± SD of data obtained in 3 separate experiments in each of which triplicate determinations were made. *$p < 0.05$, stretch vs control.

of total CaD immunoreactivity) and high amounts of l-CaD, which is indicative of their synthetic phenotype. To investigate the effect of CS on the expression of marker proteins, non-confluent VSMC cultures ($\approx 2 \times 10^4$ cells/cm^2) were subjected to CS for 4 days in the presence of 0.5% FCS, and then cell lysates were analyzed for CaD, CN, and SMM expression. CS induced a pronounced re-expression of h-CaD, whereas changes in l-CaD, CN and SMM expression were negligible (Fig. 3).

In the next set of experiments time-dependence of stretch-induced h-CaD expression was studied. A significant increase in h-CaD expression was detected on day 3 of stretching VSMC and remained unchanged until day 8 (Fig. 4). If stretching of VSMC was stopped on the fourth day, and cells were cultured until day 8 under stationary conditions, h-CaD expression in these cells reverted to control levels (Fig. 4, insert). Thus, omission of mechanical stimulation caused a restoration of low h-CaD expression level.

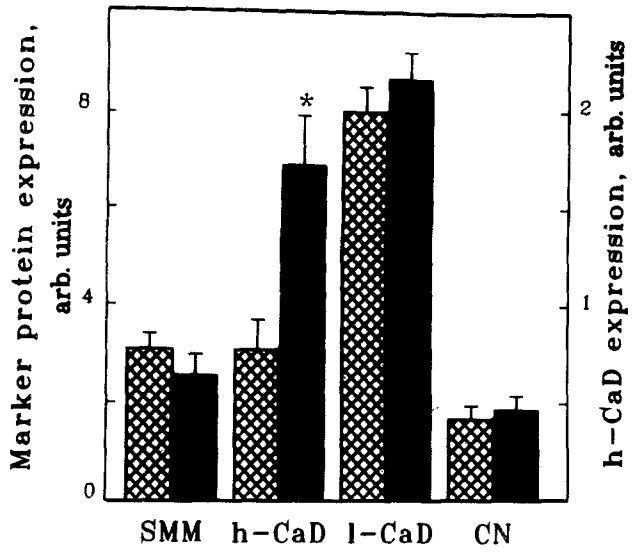

Fig. 3. Expression of VSMC contractile phenotype markers. VSMC on collagen I-coated Flex plates were cultured in the presence of 0.5% FCS for 4 days either without (hatched bars) or with (solid bars) application of CS. Marker protein expression was determined by immunoblot analysis as described in Materials and methods. SMM, smooth muscle myosin heavy chains; h-CaD, h-caldesmon; l-CaD, l-caldesmon; CN, calponin. Values given represent mean ± SD of data obtained in 3 separate experiments in each of which triplicate determinations were made. *p < 0.05.

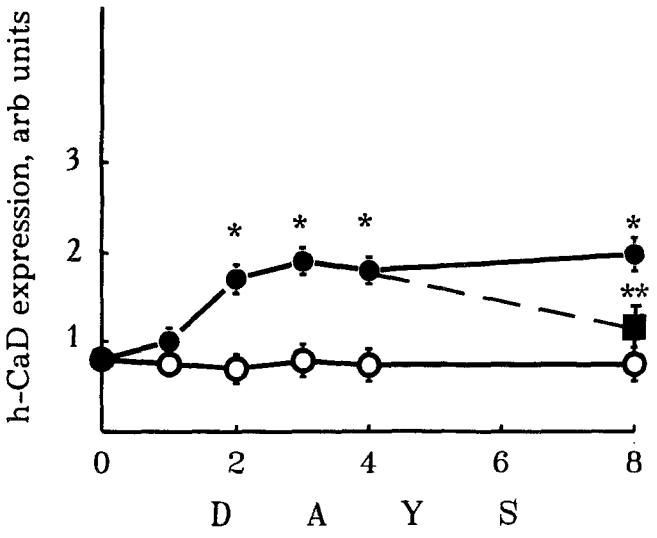

Fig. 4. Dependence of stretch-activated h-CaD expression on duration of stimulation and the influence of interruption of mechanical stimulation. VSMC on collagen I-coated Flex plates were cultured in the presence of 0.5% FCS for the indicated periods of time without (open symbols) or with (closed symbols) application of CS. Separate Flex plates stretched in the first 4 days were then placed into stationary conditions during the days 4–8 of experiment (filled box). The data points given represent mean ± SD of data obtained in 3 separate experiments in each of which quadruplicate determinations were made. *p < 0.05, stretch vs control; **p < 0.05, day 4–day 8 in stationary conditions vs stretch. The insert presents Coomassie-stained gels (lanes 1–5) and corresponding anti-caldesmon immunoblots (lanes 6–10) illustrating this experiment. VSMC were cultured for either 4 days without (lanes 1 and 6) or with CS (lanes 2 and 7), for 8 days without (lanes 3 and 8) or with CS (lanes 4 and 9), or for 4 days with stretch followed by 4 days without stretch (lanes 5 and 10).

Influence of serum and extracellular matrix components on CS-induced smooth muscle phenotype marker expression

Since serum factors and extracellular matrix components are recognized to play an important role in control of VSMC phenotype [30–32], their influence on stretch-mediated expression of smooth muscle phenotype markers was investigated. Analysis of h-CaD content in VSMC grown at various FCS concentrations and thus having different population doubling rates revealed that CS-induced h-CaD expression occurred independently of VSMC proliferation (Fig. 5). Furthermore, the effects of CS on h-CaD expression were not markedly different between cultures at subconfluency (0.5–5% FCS) or confluency (10% FCS), and the levels of CS-induced h-CaD expression were comparable for all FCS concentrations tested (Fig. 5). In control experiments on standard plastic tissue cultureware we were unable to detect alterations in h-CaD expression in VSMC either seeded at different densities (2–15 × 10^5) or cultured in the presence of different concentrations (0.5–10%) of FCS (data not shown). Taken together, these findings suggest that CS-induced h-CaD expression is independent of both serum concentration and cell density in passaged rabbit aortic VSMC.

The expression of l-CaD decreased in VSMC cultured in the presence of higher serum concentrations in stationary conditions, and this effect was even more pronounced under CS (Fig. 5). Comparison of cell enumeration data and l-CaD levels suggest a cell density-dependent decrease in l-CaD expression. There were no obvious effects of serum, cell density or CS on levels of CN and SMM expression (Fig. 5).

The extracellular matrix transduces external mechanical signals to the cell membrane via interaction with cell surface receptors that are coupled to intracellular signal transduction systems [33, 34]. To get further insight in CS-induced h-CaD expression we considered the role of various matrix components in this effect. The influence of collagen types I, IV and laminin, on CS-induced h-CaD expression was studied. For VSMC grown on a laminin substratum the CS-induced increase in h-CaD expression was approximately 2-fold higher than in VSMC grown on collagen types I and IV substrata (Fig. 6). CS-induced h-CaD expression was also observed for VSMC cultured on either poly-L-lysine, or gelatin. The levels of h-CaD on these substrata were not different from those observed on collagens (Fig. 6). In stationary cultures the lev-

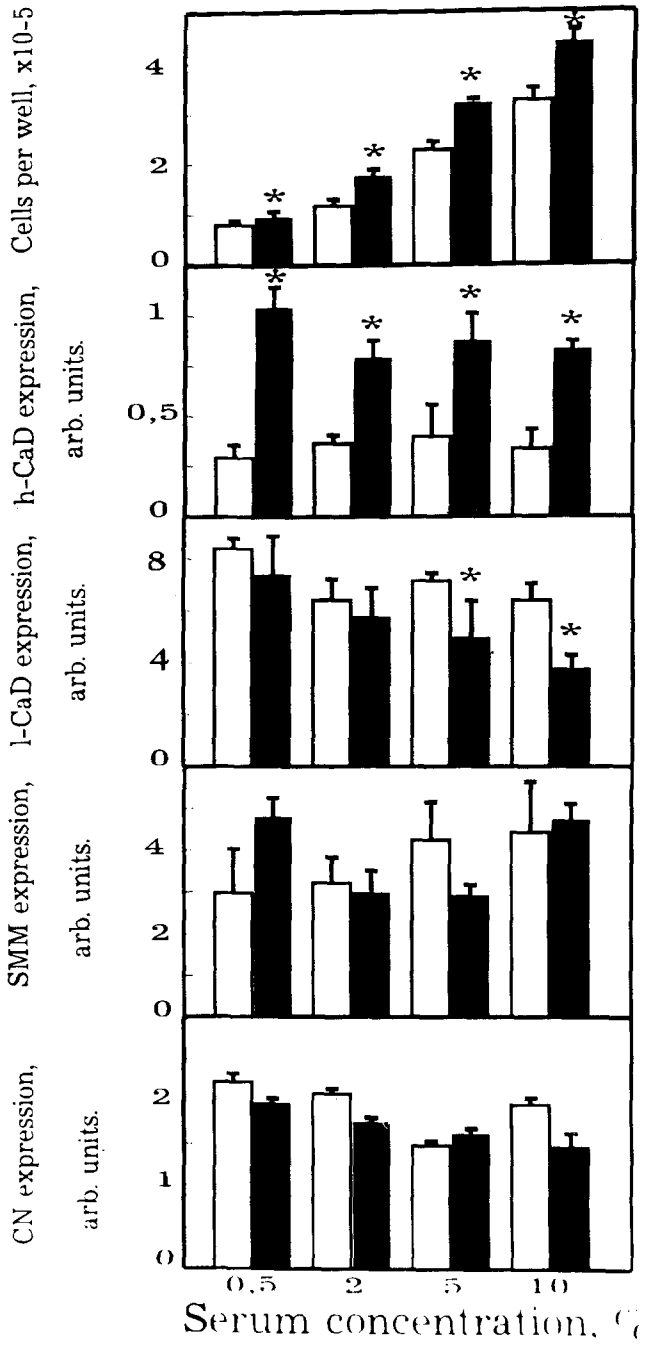

Fig. 5. Effect of serum on the contractile protein phenotype marker expression. VSMC seeded on collagen I-coated Flex plates (6×10^4 cells/well) were cultured for 6 days in the presence of the indicated concentrations of FCS without (open bars) or with (solid bars) application of CS. Cell numbers were determined and the levels of SMM, h-CaD, l-CaD and CN were simultaneously analysed. Values for cell enumeration and marker protein expression represent mean ± SD of data obtained in 3 separate experiments in each of which quadruplicate determinations were made. *$p < 0.05$.

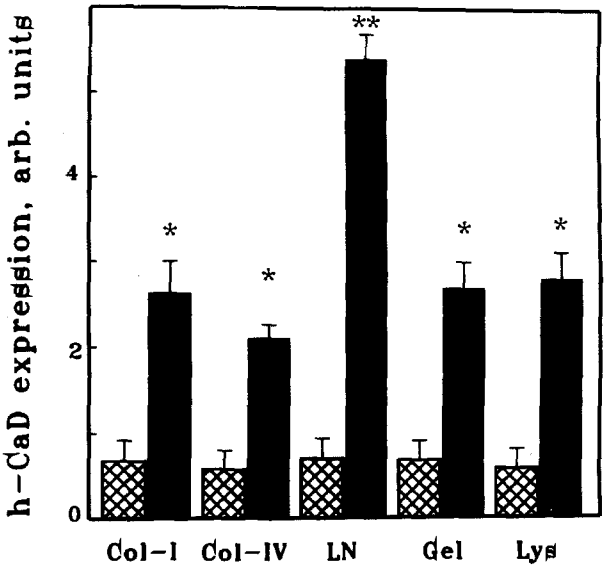

Fig. 6. Influence of different substrates on stretch-activated h-CaD expression. VSMC were cultured on various matrix-coated Flex plates and maintained in the presence of 0.5% FCS under stationary (hatched bars) or stretch (solid bars) culture conditions for 6 days. Thereafter h-CaD expression was analysed. Col-I and Col-IV, collagen types I and IV, respectively; LN, laminin; Gel, gelatin; Lys, poly-L-lysine. Values given represent mean ± SD of data obtained in 3 separate experiments in each of which quadruplicate determinations were made. *$p < 0.05$, stretch vs control: **$p < 0.05$, LN stretch vs Col-I stretch.

els of h-CaD did not vary with different substrata (data not shown).

Discussion

In the present study we used the model of VSMC cultured in mechanically active surrounding to test whether an intermittent strain affects the features associated with cell phenotype. In comparison to freshly isolated VSMC, early passaged rabbit aortic VSMC used in this work displayed proliferative activity, significantly reduced levels of the contractile phenotype protein markers SMM, h-CaD and CN, and reciprocally high level of l-CaD. We have previously shown that in modulated primary rabbit aortic smooth muscle cells and in early passaged SMM is represented by SMl isoform while SM2 isoform practically disappears in the course of modulation of primary culture [29]. These features resemble those of VSMC in locations of vessel injury. VSMC cultured in the presence of 2–10% FCS responded to CS with an increase in cell number that was detectable within 2 days of stimulation. On the contrary, in the presence of 0.5% FCS an onset of proliferation in stationary culture became evident on the fourth day in culture, suggesting a switch to an autocrine

growth stimulation by factors (e.g. PDGF) synthesized by cultured VSMC [35, 36]. Under these conditions the effect of CS on VSMC proliferation was detected after 4 days of culture suggesting that the ability of CS to promote VSMC growth depends on the proliferation status of the cells.

Increased proliferation of VSMC subjected to CS may be explained by a stimulatory effect of stretch on the expression/secretion of mitogens [16], or/and by potentiation of the effects of mitogens by CS-induced periodic Ca^{2+}-entry through stretch-activated Ca^{2+}-channels [37]. Our data demonstrating that CS significantly affects VSMC proliferation only in proliferating cultures support the potentiation mechanism. Indeed, counteraction of PDGF-induced intracellular Ca^{2+} elevation [38, 39] by Ca^{2+}-channel antagonist nifedipine inhibits VSMC proliferation [40]. Hence, an activation of Ca^{2+}-channels by stretch may add to Ca^{2+}-mobilizing action of mitogens and enhance proliferative response of VSMC. It is, however, also possible that adult VSMC used in our study have lower intrinsic rate of PDGF expression/secretion than rat embryonic VSMC used by Wilson *et al.* [16] and the lag between the onset of CS action and proliferation reflects the accumulation of sufficient amounts of mitogens in culture medium. Further experiments are required to clarify this issue.

Examination of the contractile phenotype marker proteins revealed that CS induces increased expression of h-CaD in modulated VSMC toward the levels characteristic of differentiated cells. The expression of CN and SMM was insensitive to CS in these cells. Our data demonstrate that CS-induced expression of h-CaD consistently occurs in VSMC over a wide range of FCS concentrations. It suggests that the expression of this protein may be upregulated even in profoundly modulated VSMC following appropriate stimulation. Expression of h-CaD was also insensitive to cell contact effects and was similar in VSMC seeded at different densities. These results are in apparent conflict with the dominating dogma stating a direct relationship between proliferative activity and cell dedifferentiation. However, a number of recent studies shows dissociation between these two phenomena. Corjay and co-authors [41] showed that 10% FCS being as mitogenic for cultured rat VSMC as PDGF did not alter the expression of smooth muscle alpha-actin mRNA, whereas PDGF suppressed it by 70–70%. Similar results were reported by Holycross *et al.* [30] who observed unchanged steady-state levels of smooth muscle tropomyosin mRNA in rat VSMC culture in the presence of 10% fetal bovine serum whereas 20 ng/ml PDGF having similar mitogenic effect suppressed smooth muscle tropomyosin mRNA by 80%. Desmouliere and co-workers [42] also demonstrated that FCS and rat whole blood serum reveal distinct effects on smooth muscle alpha-actin expression but equally activate cell proliferation. At the same time heparin possessed both antiproliferative and differentiating action on cultured VSMC according to classic scheme. Hence, these findings and our results suggest that cell proliferation is not necessarily associated with the coordinate downregulation of the entire differentiation program.

In contrast to h-CaD, l-CaD expression in these cells decreased with increasing cell density, as it was previously demonstrated in primary cultures of rabbit aortic VSMC [20]. However, there was no effect of CS on the expression of l-CaD.

Previous studies demonstrated that h-CaD may accumulate in BC3H1 cells following serum deprivation and confluency [43]. However, in rabbit aortic smooth muscle cells these stimuli did not affect h-CaD expression. Instead, a mechanical stimulation of VSMC in the form of CS produced significant alterations in h-CaD expression. In periodically stretched modulated VSMC increased expression of h-CaD was noticed that was reminiscent of h-CaD accumulation during VSMC differentiation in embryogenesis.

At present we have no convincing explanation why h-CaD expression, but not that of SMM or CN is regulated by CS. Perhaps, it is related to a unique actomyosin cross-linking function of h-CaD and the key role ascribed to this protein in regulation of smooth muscle contraction. Since periodic strain of arterial wall produced by blood pulsation evokes tonic contraction of the vessels there might be CS-sensitive mechanisms providing for a proper expression of the major 'tonic protein' – h-caldesmon. Recent findings of Reckless *et al.* [44] provide evidence supporting this assumption. Placing a rigid collar around carotid artery of the living animal results in a decreased h-CaD content in operated vessel.

Considering whether transmission of CS signal requires specific receptors or mere cell deformation we studied CS-induced h-CaD expression in VSMC grown on different extracellular matrices. Components of extracellular matrix interact with cell surface via integrin-type receptors and other receptors [45] which are coupled to second messenger cascades and may exert specific action on the cells [33, 34]. For instance, collagen type I promotes phenotypic modulation [31], whereas collagen type IV and laminin maintain the contractile VSMC phenotype [32]. Gelatin and poly-L-lysine represent matrices which lack biological specificity and do not affect VSMC phenotype. None of these matrices altered the level of h-CaD expression in stationary culture. However, introduction of CS leads to the increased h-CaD expression in VSMC grown on all examined substrates. These findings suggest that the principal effect of CS is not related to specific matrix receptors, and multiple matrix – receptor interactions are sufficient to deliver stretch signal to the cell membrane. Mechanical deformation may then be transmitted through a cytoskeletal network to other systems such as stretch-activated nonselective cationic channels [46], selective Ca^{2+}-channels [47] and submembrane second messenger systems [10, 48, 49].

In case of laminin we, however, observed 2–3-fold higher h-CaD expression in response to CS than on other matrices.

This corresponds well to the role of laminin in the maintenance of VSMC contractile phenotype and possibly indicates a positive 'cross-talk' between stretch-activated signalling systems and laminin receptor-associated signal transmitting pathways. These findings also suggest that the degree of CS-induced h-CaD expression may vary depending on the extracellular matrix composition. Perhaps, in the vessel wall where laminin is one of the major components of a basal membrane CS effects are more pronounced than in artificial culture conditions used in this study.

In conclusion, we observed that the repetitive mechanical deformation of cultured VSMC selectively and reversibly induces accumulation of h-CaD, a protein involved in regulation of smooth muscle contraction and considered as a reliable marker of a smooth muscle contractile phenotype. This effect was consistently reproduced in various culture conditions. Thus, mechanical stimulation of arterial vascular smooth muscle cells occurring *in vivo* may contribute to the maintenance of VSMC contractile phenotype in normal vessels and provide conditions for reversal of phenotypic modulation of VSMC in the regions of vascular injury. On the other hand, in apparent contrast with its pro-differentiating action, stretching may enhance proliferative response of VSMC. Probably, dual effects of cyclic stretch observed in our work and in literature are related to its ability to stimulate intracellular Ca^{2+} turnover and therefore to support various Ca^{2+}-dependent processes currently occurring in the cell.

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