CREG promotes a mature smooth muscle cell phenotype and reduces neointimal formation in balloon-injured rat carotid artery

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Aim We previously showed that cellular repressor of E1A-stimulated genes (CREG) is up-regulated during serum starvation-induced vascular smooth muscle cell (SMC) differentiation. The aim of this study was to determine the role of CREG in maintaining the quiescent, differentiated phenotype of SMCs both in culture and in balloon-injured rat carotid artery.

Methods and results In cultured SMCs recombinant virus-mediated CREG expression enhanced cellular differentiation, inhibited proliferation, and reduced synthesis of extracellular matrix component fibronectin. In contrast, CREG knockdown via retroviral transfer of short hairpin RNAs abrogated serum starvation-induced SMC differentiation and growth arrest. Both immunostaining and Western analysis demonstrated marked down-regulation of CREG in the vascular media after balloon injury to the rat carotid artery. Retrovirus-mediated CREG transfer to the injured artery inhibited SMC dedifferentiation and proliferation, and reduced neointimal hyperplasia.

Conclusion These results suggest that CREG participates in the maintenance of quiescent mature SMC phenotype in the arterial media by promoting SMC differentiation and growth arrest and that CREG gene transfer has therapeutic potential for vascular diseases associated with neointimal hyperplasia.

KEYWORDS
Cellular repressor of E1A-stimulated genes; Vascular smooth muscle cell; Differentiation

1. Introduction

In the normal adult artery, smooth muscle cells (SMCs) are highly specialized for contraction through which they regulate vascular tone and blood distribution. To fulfill this function, mature SMCs adopt an elongated morphology, remain quiescent in proliferation and metabolism of extracellular matrix (ECM), and express a unique repertoire of contractile proteins, agonist receptors, and ion channels. In contrast, SMCs within acute vascular lesions often display a stellate shape, express fewer contractile proteins, and are active in proliferation, migration, and ECM turnover.1–4 A large body of evidence has demonstrated that the SMCs in the neointima formed after vascular injury mainly come from the vascular media through phenotypic modulation/switching, whereby they lose their differentiation markers and gain the abilities to migrate, proliferate, and metabolize ECM. Such phenotypic modulation is fundamental to vascular adaptation and repair as well as the development of neointima hyperplasia. The latter is a key pathologic feature of atherosclerosis and angioplasty-induced restenosis.

Despite numerous studies on growth factors and cytokines that stimulate the conversion of quiescent contractile SMCs to aggressive proliferative ones after vascular injury, relatively little is known about soluble factors that promote SMC redifferentiation and growth arrest after vascular repair. The latter is an essential step of the normal healing process and is vital to limiting lesion development. We and others have shown that cellular repressor of E1A-stimulated genes (CREG), a secreted glycoprotein, is significantly up-regulated at both the mRNA and protein level during the phenotypic conversion of proliferative SMCs to quiescent differentiated ones.5,6 CREG has been shown to act as a transcription repressor to antagonize transcription activation and cellular transformation induced by the adenoviral E1A oncoprotein.7 The human homolog contains 220 amino acid residues and 3 consensus N-glycosylation sites.8,9 The secreted CREG has been shown to bind its putative receptor

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mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R) in a far Western analysis. M6P/IGF2R is a scavenger receptor that facilitates the clearance of extracellular IgGs, which are implicated in the pathogenesis of atherosclerosis and restenosis. When overexpressed in human embryonal carcinoma cells, CREG enhances cellular differentiation and inhibits cell cycle progression. However, CREG's biological activity in vascular cells and its role in vascular repair remain unknown. To test the hypothesis that CREG might participate in the maintenance of SMCs in mature phenotype in the arterial media, we performed gain- and loss-of-function analyses of CREG in cultured SMCs. We demonstrate that CREG overexpression inhibits SMC proliferation and promotes cellular differentiation, whereas its knockdown prevents serum starvation-induced SMC maturation and growth arrest. Furthermore, CREG is down-regulated in the vascular media after balloon injury to the rat carotid artery. Retrovirus-mediated CREG expression in the injured artery decreases SMC proliferation, attenuates SMC dedifferentiation, and reduces neointimal hyperplasia. These data demonstrate for the first time that CREG plays a critical role in the maintenance of the differentiated SMC phenotype and that CREG gene transfer has therapeutic potential for vascular diseases associated with neointimal thickening such restenosis after angioplasty.

2. Methods

2.1 Production of infectious retrovirus

Human CREG cDNA was subcloned from the pRC/CMV-hCREG (kindly provided by Dr Grace Gill of Harvard Medical School) into the pLNCX retroviral vector (Clontech). Green fluorescent protein (GFP) cDNA was subcloned from pEGFP-N1 (Clontech) to pLNCX. Three retroviral vectors containing short hairpin RNAs (shRNAs) targeting to the open reading frame of human CREG were purchased from Open Biosystems (M6P/IGF2R) in a far Western analysis. The cells were infected with retrovirus containing pLNCX-CREG or pLNCX-GFP retrovirus (concentrated from 2.5 Balloon injury to rat carotid arteries and retroviral transduction

Male Sprague Dawley rats (360–450 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg). The left carotid artery was injured by a 2F Fogarty balloon embolectomy catheter (Baxter) as previously described. Immediately after the balloon injury, 500 µL of Pluronic F127 (BASF Corporation) containing pLNCX-CREG or pLNCX-GFP retrovirus (concentrated from 20 mL conditioned medium by ultracentrifugation, viral titer ~10^6 pfu/mL as determined for pLNCX-GFP retrovirus), or Pluronic gel alone was placed around the carotid artery at the site of the injury. The rats were sacrificed with an overdose of pentobarbital sodium (100 mg/kg) at 2, 4, 7, 14, and 28 days (five animals for each time point) after balloon injury and retroviral infection. The uninjured right carotid arteries served as control. The experimental procedures and animal care were approved by the Institutional Animal Care and Use Committee of Shenyang Northern Hospital and Robert Wood Johnson Medical School. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).
2.6 Immunohistochemistry and morphometric analysis

The injured and the control carotid arteries were harvested at designated time points and fixed with 4% paraformaldehyde. The endogenous peroxidase activity was quenched with 0.3% H2O2 and non-specific binding sites blocked with 10% goat serum. The primary antibody was applied for 24 h at 4 °C, followed by incubation with a HRP-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Colour development was achieved with the DAB chromagen system (Dako). The aortic sections were counterstained with hematoxylin, examined on an Olympus IX70 microscope, and photographed with a CoolSNAP-Profx camera (Media Cybernetics). For morphometric analysis of neointimal formation, sections were stained with hematoxylin–eosin, photographed and analysed using image analysis software (Image-proplus 5.1, Olympus). The area of the intima, media, and adventitia was measured, and the intima/media ratio was calculated.

2.7 Statistical analysis

The data were expressed as mean ± SEM. Statistical significance between two groups was compared by an unpaired Student’s t-test. Differences between multiple groups were analysed by ANOVA.

3. Results

3.1 CREG overexpression inhibits smooth muscle cell proliferation and enhances cellular differentiation

We previously showed that CREG expression closely correlates with the proliferative and differentiation status of cultured vascular SMCs. To test the hypothesis that CREG might regulate SMC phenotypic switching from a less differentiated, proliferative state to a quiescent differentiated state, we generated stable rat vascular SMCs overexpressing CREG by infecting the cells with retroviral vector pLNCX-CREG. As demonstrated in Figure 1, pLNCX-CREG transduction caused a 3-fold increase in CREG protein expression compared with the untreated and GFP-transduced SMCs. The GFP-transduced cells assumed spread morphology when cultured in 5% FBS. In contrast, those overexpressing CREG converted to a spindle shape, a characteristic of the mature SMC phenotype. In addition, Western analysis revealed that SMC differentiation markers SM α-actin and desmin were markedly up-regulated in CREG-transduced SMCs. To examine whether the enhanced SMC differentiation might be coupled with cell proliferation, we performed FACS analysis and BrdU incorporation assay. CREG overexpression resulted in significant retention of SMCs in the G0/G1 phase when compared with the untreated and GFP-transduced cells (P < 0.01). Moreover, there was a 4-fold reduction in BrdU incorporation in CREG-overexpressing SMCs (P < 0.01 compared with untreated and GFP-expressing cells). These data demonstrate that CREG overexpression leads to cellular differentiation and growth suppression in SMCs.

SMCs enzymatically dissociated from the arterial media rapidly undergo phenotypic modulation and lose some of their differentiated characteristics when cultured on plastic in serum-containing medium, especially after subculturing. This is reflected by the disappearance of SM-MHC and increased synthesis of the ECM protein fibronectin. To determine whether CREG might inhibit the SMC dedifferentiation process, we overexpressed CREG in primary SMC cultures via adenoviral gene transfer. Western analysis of CREG-transduced cells with CREG-specific antibodies detected four bands migrating between 25–35 kDa, which likely represent differentially glycosylated CREG as treatment of cell lysates with glycosidase F downshifted the higher bands (Figure 1F, data not shown). Importantly, forced CREG expression caused a 2-fold increase of SM-MHC content (P < 0.05 compared with untreated and GFP-expressing cells) and a 3-fold reduction of cell-associated fibronectin (P < 0.01 compared with untreated and GFP-expressing cells) indicating that CREG promotes the differentiated SMC phenotype.
3.2 CREG mediates serum starvation-induced smooth muscle cell differentiation and growth arrest

We have reported that serum reduction can convert human SMCs from a proliferative, synthetic state to a quiescent, contractile state, and that CREG is up-regulated in this process. To determine whether CREG might mediate this phenotypic transition, we suppressed CREG expression in human SMCs via retroviral transfer of shRNAs that target to the open reading frame of human CREG. As shown in Figure 2, stable expression of shRNAs in the SMCs reduced CREG protein expression by ∼80% compared with the control vector-transduced cells (P < 0.01). CREG in SMC lysates displayed double bands, possibly due to different levels of glycosylation. To assess the effect of CREG knockdown on serum deprivation-induced SMC differentiation, we performed Western analysis for SMC contractile protein SMα-actin. Control SMCs cultured in serum-free medium for 3 days significantly up-regulated their SMα-actin expression (Figure 2B). In contrast, stable expression of CREG shRNAs blocked serum deprivation-induced SMα-actin expression (control vs. shRNA1 P < 0.01, control vs. shRNA2 P < 0.05). We also observed enhanced cell cycle progression from the G0/G1 phase to the S and G2/M phase after shRNA-mediated CREG suppression. Taken together, these results suggest that CREG plays a critical role in serum withdrawal-induced SMC maturation as evidenced by enhanced cellular differentiation and reduced proliferation.

3.3 CREG expression closely correlates with the proliferative and differentiation status of smooth muscle cells within the artery wall

CREG has been shown to be ubiquitously expressed in human and mouse tissues. However, its physiological functions and possible involvement in pathological processes remain unknown. We have observed enhanced CREG expression in cultured vascular SMCs as they convert from an aggressive proliferative to a quiescent mature phenotype. To determine whether CREG might also be expressed in SMCs in the arterial media and whether its expression might correlate with SMC phenotypic modulation, we introduced balloon injury to the rat carotid artery and harvested the injured arteries on days 2, 4, 7, 14, and 28 which span the period during which SMCs dedifferentiate, form an intimal lesion, and then re-differentiate as the lesion subsides. Immunohistochemistry revealed that CREG was expressed in the medial SMCs of normal arteries and was markedly down-regulated in the first week after the balloon injury, with close correlation to the SMC dedifferentiation process (reduction in SMα-actin and SM-MHC and increase in Ki67, a proliferation marker). After 7 days, the neointima started to form and CREG expression gradually restored in both the newly formed neointima and the media as the SMCs began to re-differentiate (Figure 3).

Figure 2 shRNA-mediated CREG knockdown in smooth muscle cells inhibits cellular differentiation and enhances proliferation. Human SMCs were stably transduced with short hairpin (sh) RNAs targeting to the open reading frame of human CREG (shRNA1 and shRNA2) or the empty vector (control). (A and A’) CREG secreted by the SMCs to the culture medium was analysed by immunoprecipitation (IP) followed by immunoblotting (IB) with a CREG-specific antibody. CREG in cell lysates was directly examined by IB. β-actin serves as loading control. CREG protein expression was quantified by densitometry and plotted as the ratio of CREG to β-actin. Control vs. shRNA1 and shRNA2 in both medium and the cell lysates, **P < 0.01, n = 3. (B and B’) The expression of the differentiation marker SMα-actin was determined by IB before and after serum starvation and quantified by densitometry. The expression level is depicted as the ratio of SMα-actin to β-actin. Control vs. shRNA1 **P < 0.01, control vs. shRNA2, *P < 0.05 under serum deprivation conditions, n = 3. (C) Cell cycle progression was assessed by fluorescence-activated cell sorting analysis.
Western analysis demonstrated a similar trend in the expression of CREG and contractile proteins \((P < 0.05)\). In the course of the lesion formation, CREG levels inversely correlated with SMC proliferation and dedifferentiation. These data strongly suggest that CREG is associated with the quiescent mature SMC phenotype, and that the injury-induced CREG down-regulation may facilitate SMC dedifferentiation and lesion formation.

### 3.4 Retrovirus-mediated CREG gene transfer to the balloon-injured artery inhibits neointimal formation

Since CREG induces cultured SMCs to adopt a more differentiated phenotype, and since CREG is down-regulated in the arterial media in parallel with enhanced SMC proliferation and dedifferentiation after vascular injury, we hypothesized that forced expression of CREG in the injured vessel wall might promote SMC differentiation, inhibit cell proliferation, and thus reduce neointimal formation. To test this hypothesis, we delivered CREG- or GFP-expressing retrovirus to the balloon-injured carotid artery using Pluronic gel, which has been widely employed as a carrier to deliver viral vectors and other agents to arteries.18,19 Immunohistochemistry and fluorescent microscopy detected high levels of GFP or CREG expression in the artery wall up to 28 days after balloon injury (Figure 4, data not shown). In comparison with the untreated control and GFP-transduced arteries, infection of the injured arteries with retroviral CREG markedly increased CREG levels as assessed by Western blotting \((P < 0.05)\). Histological analysis of the injured arteries revealed that retrovirus-mediated CREG transfer significantly decreased the neointimal area (CREG vs. GFP or untreated control group, \(P < 0.05\)).
3.5 Retrovirus-mediated CREG gene transfer to the injured artery inhibits smooth muscle cell dedifferentiation, proliferation, and extracellular signal-related kinase activation

Numerous studies have suggested that neointimal hyperplasia after acute vascular injury results from the dedifferentiation of SMCs in the media and their subsequent migration to and proliferation in the intima. To determine whether retroviral transfer of CREG to the injured arterial wall might inhibit SMC proliferation and enhance cellular differentiation, we examined the expression of SMC differentiation markers SM-MHC and SM α-actin by Western blotting. Tissue homogenates of 4-day post-injury arteries had a 50% reduction of SM-MHC and SM α-actin in the untreated and GFP-transduced vessels (CREG vs. GFP or untreated control group, \( P < 0.01 \)) (Figure 6a, data not shown). Of note, the mature type of SM-MHC SM2 was completely disappeared. In contrast, CREG transduction attenuated the reduction of these contractile proteins in the injured artery. Furthermore, SMC proliferation in CREG-transduced arteries was significantly inhibited as evidenced by reduced Ki67-positive cells in the neointima and the media. These results suggest that CREG participates in the maintenance of the quiescent, mature SMC phenotype in the vascular media and that CREG gene transfer may have therapeutic potential for vascular diseases associated with SMC dedifferentiation and neointimal hyperplasia.

To determine whether the reduced neointimal formation by CREG transduction might also result from induction of apoptosis, we immunostained 4-day post-injury arteries for cleaved (active) caspase-3, a marker for apoptosis. As demonstrated in Figure 6c, many positive cells were
identified in the media layer and the luminal surface of untreated or GFP-transduced arteries. Surprisingly, CREG transduction reduced apoptosis in the arterial wall after balloon injury. These data indicate that apoptosis does not account for reduced neointimal formation after CREG transduction.

Mitogen-activated protein kinase (MAPK) has been implicated in the development of neointima after acute vascular injury. To test whether CREG might modulate extracellular signal-related kinases (ERK) activity in injured arteries, we performed Western analysis using an antibody that specifically reacts with active, tyrosine-phosphorylated ERK1 and 2. Balloon injury to the carotid artery induced marked activation of ERK1 and 2, which gradually returned to the normal level after 2 weeks. Retrovirus-mediated CREG transfer inhibited ERK activation as compared to the GFP control (P < 0.01) (Figure 6D). These results suggest that CREG-mediated inhibition of ERK activation after vascular injury may contribute to the reduced SMC proliferation and neointima hyperplasia.

4. Discussion

CREG is a ubiquitously expressed protein in mammals. However its functions in the vasculature under normal and pathologic conditions are unknown. In this study, we demonstrate, for the first time, that overexpression of CREG inhibits proliferation and enhances differentiation in cultured vascular SMCs. Moreover, shRNA-mediated CREG knockdown abrogates serum starvation-induced SMC maturation and growth arrest. In balloon-injured rat carotid artery, CREG expression is down-regulated in the vascular media in parallel with SMC contractile proteins SM α-actin and SM-MHCs. Forced expression of CREG in the injured arteries inhibits SMC proliferation, attenuates SMC dedifferentiation, and reduced neointimal formation. Retrovirus-mediated CREG expression also inhibits the activation of MAPK pathway, which may underlie the anti-proliferative effect of CREG. Taken together, these results indicate that CREG plays a critical role in the maintenance of SMCs in a mature, growth quiescent state in the normal artery and that CREG down-regulation contributes to neointimal hyperplasia after vascular injury.

Under physiological conditions, SMCs in the normal adult artery are maintained in a quiescent contractile state. Similarly, SMCs switch from a migratory, proliferative, and synthetic phenotype to a non-proliferative and contractile one in the process of vascular maturation during embryonic development as well as in the resolution phase of vascular repair. However, it is largely unknown how this phenotypic switching is attained and how the quiescent contractile state is maintained in these processes. Given that a number of growth factors including platelet-derived growth factors, IGFs, and transforming growth factors have been implicated in the conversion of SMCs from contractile to synthetic phenotype, it is likely that growth factor withdrawal may lead to the acquisition of the quiescent contractile state. Another possibility is the active secretion of soluble factors and ECM proteins that stimulate the phenotypic switching. Small et al. has recently shown that CREG mRNA is significantly up-regulated during the conversion of a human vascular SMC line from non-contractile to contractile state. We have also shown that in serum starvation-induced differentiation of confluent vascular SMCs, CREG protein expression is increased in parallel with smooth muscle contractile proteins SM α-actin and calponin. In the present study, we utilized both gain- and loss-of function analysis to determine the biological activity of CREG in vascular SMCs and its role in serum starvation-induced SMC differentiation and growth suppression. We found that CREG overexpression converted SMCs from a spread to a spindle shape and enhanced the expression of SM α-actin, desmin, and SM-MHC, indicating that CREG promotes a quiescent mature phenotype in SMCs. The knockdown study has confirmed this notion, demonstrating that CREG suppression attenuated serum starvation-induced SM α-actin expression and growth arrest. This result suggests that CREG mediates serum starvation-induced SMC maturation. Our data are in agreement with the observations that CREG, when overexpressed, enhanced human embryonic carcinoma cell differentiation and inhibited the proliferation of rat neonatal cardiomyocytes. Recently we observed that CREG knockdown promoted cell proliferation in NIH 3T3 fibroblasts (data not shown). Since CREG is highly expressed in many adult tissues, these findings support the hypothesis that it may participate in the induction and maintenance of the quiescent, differentiated cell phenotype in various tissues.

The in vivo function of CREG has not been reported. To determine whether CREG might also regulate SMC phenotype modulation in the artery wall, we introduced balloon injury to the rat carotid artery and examined CREG expression by immunohistochemistry and Western blot analysis. We found that CREG was highly expressed in the SMCs of the arterial media and the endothelium. Its expression was significantly decreased as SMCs dedifferentiated and proliferated in the first week after balloon injury, and was restored to the normal levels as SMCs re-differentiated and the lesion became quiescent. Note, retroviral transfer of CREG into the injured artery wall inhibited SMC dedifferentiation and proliferation and reduced neointimal hyperplasia. These data suggest that CREG may play a critical role in maintaining the medial SMCs in a quiescent contractile state in normal adult artery and that CREG gene/protein transfer has therapeutic potential for arterial restenosis after angioplasty. One caveat is that in patients with restenosis intimal hyperplasia often develops on top of atherosclerotic lesions in which SMCs may respond differently to injury from what modeled in this study. In the future, it is desirable to examine the role of CREG in SMC phenotypic modulation in animal models of acute vascular injury combined with atherosclerosis.

Our data demonstrate that retrovirus-mediated gene transfer leads to the restoration of CREG expression in injured rat carotid arteries and a significant reduction, but not totally absence of neointimal formation. These results suggest that overexpression of CREG alone is not sufficient to completely block neointima hyperplasia. One possible explanation for this is that the growth suppression induced by CREG overexpression is not enough to override the effect of growth-stimulating factors released after vascular injury. Another possibility is that growth inhibitors other than CREG may also be down-regulated after vascular injury. Thirdly, reduction of CREG receptor(s) or
ligand-receptor binding may also account for the incomplete inhibition of neointima formation.

CREG was initially postulated as a transcription repressor for the oncoprotein E1A-activated genes. Later it was found to be a secreted glycoprotein that binds to M6P/IGF2R in a far-Western analysis. M6P/IGF2R has no intrinsic signalling transduction activity but acts as a scavenger receptor. After binding to IGF-II and to a lesser extent IGF-I, the receptor is internalized and degraded together with the ligands. Therefore, the IGF2R counteracts the mitogenic effect of IGFS and thus negatively regulates cell growth. IGFS are potent activators of MAPK pathways that mediate IGF-stimulated cell proliferation, and have been implicated in the pathogenesis of atherosclerosis and restenosis after angioplasty. In this study, we observed strong activation of ERK1/2 immediately following vascular injury, which gradually returned to the baseline after 2 weeks. Importantly, CREG gene transfer to the injured carotid artery significantly inhibited ERK activation. This result suggests that the inhibition of ERK activation may be responsible for CREG-induced SMC growth suppression and neointimal reduction. Recently, we detected nuclear localization of CREG in several mouse tissues by immunohistochemistry (data not shown). This finding revitalizes the hypothesis that CREG may act as a transcription repressor of proliferation-related genes. Whether CREG functions as a transcription repressor in the nucleus or a soluble ligand for membrane receptor M6P/IGF2R is currently under active investigation.

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References