Laboratory Research

Polyclonal antibody production and expression of CREG protein in human vascular smooth muscle cells

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Objectives The cellular repressor of E1A-activated genes (CREG), a novel gene, was recently found to play a role in inhibiting cell growth and promoting cell differentiation. The purpose of this study was to obtain antibody against CREG protein and to study the expression of CREG protein in human internal thoracic artery cells (HITASY) which express different patterns of differentiation markers after serum withdrawal. Methods The open reading frame of CREG gene sequence was amplified by PCR and cloned into the pGEX-4T-1 vector. Glutathione-S-transferase (GST)-CREG fusion protein was expressed in E.coli BL21 and purified from inclusion bodies by Sephacryl S-200 chromatography. Rabbits were immunized with the purified GST-CREG protein. Western blot analysis detected the expression of smooth muscle-specific markers (SM α-actin, Calponin). The localization of CREG protein was examined with immunohistochemistry staining and the protein expression level was analyzed by Western blot in HITASY cells after serum removal. Results It was confirmed by using endonuclease digesting and DNA sequencing that the PCR product of CREG was correctly inserted into the vector. The GST-CREG protein was purified with gel filtration chromatography. Polyclonal antibody against GST-CREG was obtained from rabbits. CREG protein immunohistochemistry staining displayed a perinuclear distribution in the cytoplasm of HITASY cells. Results from Western blot suggested that comparing with the untreated cells upregulation of CREG protein, SM α-actin and Calponin is induced respectively in HITASY cells after serum deprivation. Conclusions The specificity of polyclonal antibody against CREG was confirmed. Using this antibody, the changes of CREG protein expression was observed in the process of phenotypic modulation of HITASY cells. These results provide basic understanding on the relationship of CREG gene with the cell phenotypic conversion. (J Geriatr Cardiol 2005,2(2):118-122).

Key Words E1A cellular repressor; polyclonal antibody; vascular smooth muscle cells; differentiation

Introduction

Vascular smooth muscle cells (VSMCs) perform diverse functions that may be categorized as differentiation (contractile phenotype) and dedifferentiation (synthetic phenotype).1 After arterial injury, multiple growth factors and cytokine genes are induced in the promotion of phenotypic modulation, migration and proliferation of VSMCs. In consequence, the expression of many growth factor genes leads to a final, common endpoint: intimal hyperplasia. Therefore, a single inhibitor of one growth factor or its receptor is unlikely to have significant effects on arterial repair or preventing intimal hyperplasia. This conclusion has led investigators to pursue regulators of cell cycle as well as potential inhibitors of cell growth.

The adenovirus E1A protein both activates and represses gene expression to promote cellular proliferation and inhibit differentiation through regulating cell cycle.2,3 The cellular repressor of E1A-stimulated gene (CREG), a novel gene, shares limited sequence similarity with E1A and binds both the general transcription factor TBP and the tumor suppressor pRb in vitro. It has been hypothesized that CREG may function in inhibiting cell growth and/or promoting differentiation.4,5 Interestingly, we have observed that the level of CREG mRNA increased in differentiation of human internal thoracic artery cells (HITASY) but reduced or even became negative in dedifferentiation.6 However, it is equivocal whether or not CREG protein is expressed and changed in VSMCs after serum withdrawal. Because the CREG antibody is not commercially available at present, we firstly constructed the pGEX-4T-1/CREG vector, produced the anti-CREG antibody, and then observed the relationship between the expression of CREG protein and the phenotypic conversion of VSMCs.
Materials and methods

Materials

Sepacryl S-200, Sepharose 4B affinity column and pGEX-4T-1 [a glutathione-S-transferase (GST) fusion protein expression vector] were provided by Pharmacia (Peapack, NEW JERSEY, USA). Platinum Pfx DNA polymerase kit was from Invitrogen (Carlsbad, California, USA). BL21 (DE3) pLysS competent cells for expression were obtained from Promega Corporation, USA. HRP-conjugated goat anti-rabbit IgG, anti-smooth muscle (SM) α-actin and anti-Calponin monoclonal antibodies were purchased from Jackson ImmunoResearch. HITASY cells, HRP-conjugated control cells for expression were obtained from Promega from Invitrogen. Expression vector were provided by Pharmacia (Peapack, AAGGATCCATGGCCGGGCTATCCCGC-3′ and 5′-GGCGATTCCTCAGCACTTGTGACATATAATCATC-3′ (restriction sites for BamH1 and EcoRl underlined). The PCR reactions used were Pfx DNA polymerase, primers, template of CREG cDNA and other components. The PCR amplification was performed with a routine procedure. After detected by 1.0% agarose gel electrophoresis, the purified PCR product was digested with BamH1 and EcoRI (TaKaRa Biotechnology Co., Ltd.), and ligated to pGEX-4T-1 vector digested with the same enzymes. The ligation mixtures were used to transform BL21 competent cells, and the desired clone was identified as white colonies on isopropyl-B-D-thiogalactopyranoside (IPTG)/ X-GAL plate and cultured in LB medium overnight. It was subsequently confirmed by sequencing (TaKaRa Biotechnology Co., Ltd.). The result of sequencing will be compared to sequences in GenBank using the BLAST.

Expression and purification of GST-CREG fusion protein

After optimization, the fresh overnight culture of bacteria, containing the recombinant plasmid, was diluted 1:100 in 500ml LB medium and induced with 0.1mmol/L IPTG for another 4 hours. After centrifugation, the pellets of bacteria were incubated with 10ml buffer A (STE containing 0.3g/L lysozyme) on ice for 30 min. Lysis of the bacteria was achieved by additional sonication. The bulk of the GST-CREG protein was selectively solubilized from the inclusion bodies by vortexing with 10ml buffer B (STE containing 8mol/L urea). The solubilized GST-CREG was loaded to a Sepacryl S-200 gel column (100cm×2.6cm) equilibrated and eluted by buffer B. The equilibration and elution processes were monitored by Biologic and BioFrac system (Bio-Rad). The flow rate was 0.5ml/min, and the 3ml fractions were collected. Elution was monitored at wavelength 254nm and 280nm. The peak fractions which showed a single GST-CREG protein band on SDS-PAGE, were subsequently pooled together. After gel filtration, the purified GST-CREG protein was dialyzed against a descending gradient of urea in buffer C (10mmol/L Tris-Cl, pH 8.0, 50mmol/L NaCl) at 4°C. At all stages of purification, the protein concentrations were determined by the Bradford method using bovine serum albumin as a standard.

Production and detection of polyclonal antibody against CREG protein

Polyclonal rabbit antibody towards the prepared GST-CREG fusion protein was raised by administering 250 µg/mL of the recombinant protein into New Zealand breed rabbits. The fundamental and booster immunity procedures and the enzyme-linked immunosorbent assay (ELISA) were after Harlow and Lane. ELISA was performed to check the antibody titer before serum collection. Antisera were purified with absorption onto GST/glutathione-Sepharose beads and the consequent separation from the beads. A modified Western blot procedure described by Sambrook was used. Briefly, 1.0 µg GST protein and 1.0µg purified GST-CREG protein were electrophoreses in 10% SDS-PAGE, transferred to PVDF membranes, and then incubated with the purified polyclonal antibody (1:1 500) respectively. The HRP-conjugated goat IgG against rabbit (1:3 000) was used as secondary antibody. The enhanced chemiluminescence Western blotting reagent kit (Amersham Pharmacia Biotech) was used as the detecting system.

Cell culture and in vitro differentiation

HITASY cells were grown in Dulbecco’s Modified Eagle’s Medium(DMEM) containing 10% fetal bovine serum (FBS), 5mmol/L penicillin and 5mmol/L streptomycin. For differentiation, HITASY cells were cultured in the DMEM without FBS for 0-7 days.

Immunohistochemical staining and Western blot analysis of HITASY cells

The distribution and expression level of CREG protein in cells was examined with immunohistochemistry staining. After serum withdrawal from culture medium for 3 days, HITASY cell coverslips were fixed with 4% paraformaldehyde at room temperature for 30 min. The procedures of permeabilization and blocking were after the standard methods. Coverslips were incubated with anti-CREG antibody (1:100, diluted in PBS) at 4°C overnight and then were washed three times with PBS. HRP-conjugated goat IgG against rabbit (1:200) was used as secondary antibody. The changes of expression levels of SM α-actin, Calponin and CREG protein were analyzed with Western blot, respectively. Cell lysates of HITASY cells were harvested before and during the 1st day to 7th days of serum withdrawal. Equal amounts of protein were separated with 12% SDS-PAGE. Primary antibodies used were the following: anti-SM α-actin antibody (1:2 000), anti-Calponin antibody (1:2 000) and anti-CREG polyclonal antibody (1:1 500) respectively.
Results

Construction of expression vector pGEX-4T-1/CREG

The process of constructing recombinant vector was shown in Fig. 1a. The PCR-amplified open reading frame sequence of CREG, about 670bp, was in good agreement with the expected value (Fig. 1b). Successful selection of the right clone containing the amplified fragment was achieved by blue/white selection and restriction analysis (Fig. 1c). DNA sequencing and BLAST analysis revealed that the nucleotide sequences completely matched with the published sequence in GenBank database.

Expression and purification of the GST-CREG

A 47 kDa GST-CREG Fusion protein was induced in E.coli BL21 by IPTG as shown in Fig. 2a. Under the culture and induction condition used, GST-CREG protein accounted for 30% of the total bacterial protein which was analyzed with 10% SDS-PAGE and the gel analysis software (Bio-Rad). After denatured with 8mmol/L urea, GST-CREG appeared in the soluble supernatant. The final supernatant was used to further purify the expressed protein using Sephacryl S-200 gel filtration, and a typical elution pattern is shown in Fig. 2b. SDS-PAGE result showed that one major peak containing the 47 kDa protein was the GST-CREG fusion protein, and the purity of the protein was estimated to be about 90%, as shown in Fig. 2c.

Fig. 1. a. Construction scheme of pGEX-4T-1/CREG expression vector; b. Electrophoretic analysis of CREG PCR products The amplification of CREG DNA fragment was performed in a 50 μl reaction mixture. A 20μl sample was subjected to electrophoresis on 1.0% agarose gel. M: DL2000 DNA markers (1 bp DNA ladder; left lane); CREG PCR products (670bp, right lane); c. Identification of recombinant plasmid pGEX-4T-1/CREG by digesting with BamHIII and EcoRI. A 15μl sample was subjected to electrophoresis in 1.0% agarose gel. Left lane; DL2000 DNA marker; Right lane; pGEX-4T-1 (upper), CREG (lower).

Fig. 2. a. SDS-PAGE of GST-CREG fusion protein. Lane M: protein marker; Lane 1: crude lysate of BL21 transformed by pGEX-4T-1/CREG without IPTG induction; Lane 2: crude lysate of BL21 transformed by pGEX-4T-1 with 0.1mmol/L IPTG induction; Lanes 3, 4: crude lysate of BL21 transformed by pGEX-4T-1/CREG with 0.1mmol/L and 0.5mmol/L IPTG induction, respectively; b. Final purification of GST-CREG through Sephacyrl S-200 gel filtration chromatography. Supernatant of GST-CREG was solubilized in 10ml of 8mmol/L urea in buffer C and loaded onto a gel filtration column. Fractions of 3ml were collected and the peaks were pooled separately. A single peak of the GST-CREG elution is shown (arrow); c. SDS-PAGE analysis of GST-CREG Lane M: protein marker; lanes 1,2,3: GST-CREG after purification with Sephacyrl S-200 (different fractions from the peak pointed with arrows); lane 4: crude lysate of BL21 transformed by pGEX-4T-U/CREG with 0.1mmol/L IPTG induction.
Preparation and identification of the CREG antibody

CREG antibody was obtained. ELISA tests indicated that the titer was above 1:100,000. Western blot showed that the antibody did not react with GST protein. It only reacted with the purified GST-CREG (Fig.3). These results confirmed that the antibody was specifically towards CREG part of the recombinant protein. The preimmune rabbit serum showed no reactivity with the fusion protein (data not shown).

Expression of SM α-actin and calponin in HITASY cells

To investigate the relationship between attainment of a contractile phenotype and expression of biochemical markers of VSMC differentiation, Western blot analysis was performed on HITASY cells. Cell lysates were harvested before and after serum withdrawal for 16 hours and 1, 2, 3, 5 and 7 days. As shown in Fig 4, the contractile apparatus proteins SM α-actin and Calponin were present in HITASY cells before withdrawal of serum. After serum removal, there was a gradual increase of the expression levels of SM α-actin and Calponin respectively.

Expression of CREG protein in HITASY cells

As shown in figure 5a, immunohistochemistry staining showed that CREG protein was expressed in HITASY cells after serum withdrawal for 3 days but was not positive in HITASY cells before serum withdrawal. The immunohistochemical CREG staining was distributed in the cytoplasm in a perinuclear pattern. Western blot further revealed that serum withdrawal induced remarkable increase of CREG protein expression level and that the cellular CREG reached a peak by 3–4 days and then gradually reduced by 5–7 days after serum withdrawal (Fig.5b).

Discussion

CREG is a small molecule containing 220 amino acids. It is a secreted glycoprotein which is conservative through evolution. Human CREG is 77% identical to mouse, and 31% identical to the Drosophila homology. In adult tissues CREG mRNA is broadly expressed and is regulated during embryonic development. CREG mRNA was also demonstrated in human cell lines.5,5,9 To explore the role of CREG in VSMCs, polyclonal antibody against CREG was conducted. In this work the important step for producing high titer antibodies was to use GST-CREG fusion protein as immunogen since GST fusion (1) may improve the immunogenic effect of CREG in rabbit body and (2) can avoid degradation by the proteases of cell. It was known that over-expression of recombinant proteins in E. coli often resulted in the formation of insoluble and inactive material such as inclusion bodies. The GST-CREG fusion
protein was also found to be included in inclusion bodies during transformation in the bacteria. Further purification with Sephacryl S-200 gel filtration column after denatured by high concentration urea was necessary. The elution produced a single peak and the pooled protein under the peak was near homogeneity (~90% pure) as supported from results of SDS-PAGE analysis. The specificity and high reactivity of CREG antibody was confirmed with evidence from immunohistochemistry as well as Western blot. Previous report from Gill’s group was the only one that obtained CREG antibody through immunization rabbits. However, the process about preparation of the antibody was not particularly described. We have described here a simple and efficient method for obtaining of the CREG polyclonal antibody.

Using the same CREG polyclonal antibody, we have found that the expression of CREG protein in VSMCs of rat significantly changed after balloon injury. In the present work, serum withdrawal induced remarkable increase of CREG protein expression in HITASY cells as shown in Western blot analysis. In general consideration, the upregulation of the well-known marker proteins of smooth muscle cells (SMCs) SM α-actin and Calponin always indicates that VSMCs are in differentiation or contractile phenotype. In agreement to the previous studies the present work demonstrated that HITASY cells could switch synthetic phenotype to contractile phenotype during serum withdrawal. Here we hypothesize that the changes in CREG protein expression may take place in connection with the process of phenotypic modulation of VSMCs in vitro. It is necessary in future studies to understand the further function of CREG.

References