Complement 3 Is Involved in the Synthetic Phenotype and Exaggerated Growth of Vascular Smooth Muscle Cells From Spontaneously Hypertensive Rats

Zhi-Hong Lin, Noboru Fukuda, Xue-Qing Jin, En-Hui Yao, Takahiro Ueno, Morito Endo, Satoshi Saito, Koichi Matsumoto, Hideo Mugishima

Abstract—Vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) show the synthetic phenotype and exaggerated growth in comparison with VSMCs from normotensive Wistar-Kyoto (WKY) rats. We investigated genes associated with the synthetic phenotype and exaggerated growth of VSMCs from SHR by microarray. Expression of 1300 transcripts was evaluated by microarray with total mRNA extracted from mid-layer aortic smooth muscle of 3-week-old SHR/Izumo and WKY/Izumo rats. mRNAs encoding sodium-dependent neurotransmitter transporter, epidermal growth factor precursor, EEF2, leptin receptor long-isoform b, clathrin assembly protein short form, and precomplement 3 (pre-pro-C3) were expressed only in aortic smooth muscle from SHR by microarray and by reverse-transcription polymerase chain reaction analysis. Pre-pro-C3 mRNA was detected only in cultured VSMCs from SHR. Exogenous C3 changed VSMCs to the synthetic phenotype. Antisense oligodeoxynucleotides (ODN) to C3 reduced the higher level of DNA synthesis in VSMCs from SHR. Antisense ODN to C3 increased expression of SM22/H9251 mRNA and decreased expression of osteopontin and matrix Gla mRNAs. It also decreased expression of growth factor mRNAs in VSMCs from SHR. In conclusion, we have shown that C3, independent of other complement molecules, has direct effects on the phenotype of VSMCs and stimulates growth of these cells. C3 is produced only by VSMCs from SHR. Therefore, C3 may be the gene underlying the synthetic phenotype and exaggerated growth of VSMCs from SHR. C3 may be a new target for the treatment of hypertension. (Hypertension. 2004;44:42-47.)

Key Words: hypertrophy ■ remodeling ■ rats ■ muscle, smooth, vascular

Spontaneously hypertensive rats (SHRs), an animal model of essential hypertension, show exaggerated growth of cardiovascular organs in comparison with normotensive Wistar-Kyoto (WKY) rats. Enhanced DNA synthesis and organ hypertrophy before the elevation of blood pressure have been described in SHRs. In addition, SHR-derived vascular smooth muscle cells (VSMCs) in culture show the exaggerated growth in comparison to cells from WKY rats. We have previously shown that SHR-derived VSMCs produce angiotensin II (Ang II) in homogenous cultures. We have reported that the mechanism underlying this enhanced generation of Ang II in VSMCs from SHRs appears to be the change from the contractile to the synthetic phenotype with increases in numbers of cytosolic organelles in comparison with VSMCs from WKY rats. We hypothesized that genetic abnormalities are involved in the exaggerated growth and synthetic phenotype of VSMCs from SHRs. We investigated the responsible genes and found that the mRNA encoding complement 3 (C3) is expressed only in VSMCs from SHR and is associated with both the synthetic phenotype and exaggerated growth.

Methods

Extraction of mRNA From Aortic Smooth Muscle and Microarray Analysis

The aorta were removed from 3-week-old male SHR/Izumo and WKY/Izumo rats (SHR Corporation, Funabashi, Chiba, Japan). mRNA was extracted directly with oligo-dT-cellulose with the quick prep micro mRNA purification kit (Amersham Pharmacia Biotech) per the manufacturer’s instructions. Experiments with GeneChips (Affymetrix, Santa Clara, Calif) were performed according to the manufacturer’s instructions. The biotin-labeled cRNA was injected into a Rat Neurobiology U34 Probe Array Cartridge, which contains probe sets for 1322 rat genes for hybridization.

Cell Culture

VSMCs were obtained by explant from aortas of 3-week-old male WKY/Izumo rats and SHR/Izumo. VSMCs were maintained in Dulbecco modified Eagle medium (DMEM) with 10% calf serum (Gibco Life Technologies), 100 U/mL penicillin, and 100 mg/mL streptomycin. Experiments were performed on cells between passages 5 and 10.
Ranking of Transcripts Ratio in VSMCs From SHR and WKY Rats

<table>
<thead>
<tr>
<th>N</th>
<th>Description</th>
<th>SHR/WKY</th>
<th>SHR Value</th>
<th>WKY Value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sodium-dependent neurotransmitter transporter</td>
<td>∞</td>
<td>177±18</td>
<td>0±0</td>
<td>0.011</td>
</tr>
<tr>
<td>2</td>
<td>Epidermal growth factor precursor</td>
<td>∞</td>
<td>1826±191</td>
<td>0±0</td>
<td>0.011</td>
</tr>
<tr>
<td>3</td>
<td>UI-R-E1-99-h-01–0-U*</td>
<td>∞</td>
<td>305±57</td>
<td>0±0</td>
<td>0.033</td>
</tr>
<tr>
<td>4</td>
<td>Leptin receptor isoform b (OB-Rb) mRNA</td>
<td>∞</td>
<td>719±148</td>
<td>0±0</td>
<td>0.04</td>
</tr>
<tr>
<td>5</td>
<td>Clathrin assembly protein short form (CALM)</td>
<td>∞</td>
<td>221±46</td>
<td>0±0</td>
<td>0.04</td>
</tr>
<tr>
<td>6</td>
<td>Preprocollagen 3</td>
<td>∞</td>
<td>810±175</td>
<td>0±0</td>
<td>0.044</td>
</tr>
<tr>
<td>7</td>
<td>J04423 E*</td>
<td>9.06</td>
<td>1154±63</td>
<td>127±127</td>
<td>0.027</td>
</tr>
<tr>
<td>8</td>
<td>Activity and neurotransmitter-induced early gene</td>
<td>7.85</td>
<td>3384±623</td>
<td>431±75</td>
<td>0.005</td>
</tr>
<tr>
<td>9</td>
<td>Phosphoinositide 3-kinase regulatory subunit p85α</td>
<td>3.49</td>
<td>1804±250</td>
<td>517±267</td>
<td>0.033</td>
</tr>
<tr>
<td>10</td>
<td>Inhibitor of apoptosis protein</td>
<td>2.9</td>
<td>4483±721</td>
<td>1544±783</td>
<td>0.009</td>
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<tr>
<td>11</td>
<td>Dopamine D3 receptor</td>
<td>2.84</td>
<td>1045±161</td>
<td>367±201</td>
<td>0.036</td>
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<tr>
<td>12</td>
<td>Type-1 astrocyte and olfactory- limbic associated protein AT1</td>
<td>2.51</td>
<td>4012±1649</td>
<td>1599±1601</td>
<td>0.036</td>
</tr>
<tr>
<td>13</td>
<td>Dahl/Rapp S (salt-sensitive) inducible nitric oxide synthase NOS2</td>
<td>1.45</td>
<td>8159±570</td>
<td>5602±783</td>
<td>0.017</td>
</tr>
</tbody>
</table>

*Unknown gene.

**Determination of DNA Synthesis and Cell Numbers**

Quiescent VSMCs were incubated with DMEM containing [3H]thymidine (0.5 μCi/mL) (NEN Research Products) for 2 hours. Each well was then washed with 1 mL of 150 mmol/L NaCl, and the cells were fixed in 1 mL of ethanol:acid (3:1) solution for 10 minutes. Acid-insoluble material was precipitated with 1 mL of ice-cold perchloric acids, and DNA was extracted into 1.5 mL perchloric acid by heating at 90°C for 20 minutes.

VSMCs were trypsinized with 0.05% trypsin at 24, 48, and 72 hours after inoculation, and cell numbers were counted with a coulter counter (Coulter Electronics Ltd).

**Reverse-Transcription Polymerase Chain Reaction Analysis**

mRNA was extracted from aortic mid-layer smooth muscle and cultured VSMCs from 3-week-old WKY rats and SHRs. Aliquots of mRNA were reverse-transcribed into single-stranded cdNA by incubation with avian myeloblastoma virus reverse-transcriptase (Takara Biochemicals). Diluted cdNA products were then subjected to polymerase chain reaction (PCR). The primers used to amplify sodium-dependent neurotransmitter transporter (SDNT), epidermal growth factor (EGF) precursor, leptin receptor long isoform b (OB-Rb), clathrin assembly protein short form (CALM), preprocollagen 3 (pre-pro-C3), SM22α, osteopontin, matrix Gla, platelet-derived growth factor (PDGF)-A chain, basic fibroblast growth factor (bFGF), and transforming growth factor-β1 (TGF-β1) are listed in Table I (available in an online supplement at http://www.hypertensionaha.org). Human 18S ribosomal RNA was used as an internal control. PCR was performed for 30 cycles according to the profiles shown in Table II (available in an online supplement at http://www.hypertensionaha.org). PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus), and products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized by ultraviolet illumination.

**Western Blot Analysis for C3 Protein in VSMCs In Vitro**

VSMCs (5×10^6 cells/cm²) were disrupted with lysis buffer (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 0.02% sodium azide, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1% Triton X-100). Total proteins were extracted and purified with 100 μL of chloroform and 400 μL of methanol. Protein samples were boiled for 3 minutes and subjected to electrophoresis on 8% polyacrylamide gels and then blotted onto nitrocellulose membranes (BioRad Laboratories). Blots were incubated with rabbit polyclonal antibody specific for C3 (Santa Cruz) or mouse monoclonal antibody specific for α-tubulin (Sigma) as an internal control, and were then incubated with goat antirabbit IgG or goat anti-mouse IgG (BioRad). Immunocomplexes were detected by enhanced chemiluminescence (ECL, Amersham).

**Synthesis of Antisense Oligodeoxynucleotides**

An appropriate antisense oligodeoxynucleotides (ODN) was identified by sequence homology analyses of the 19 nucleotides surrounding the initiation codon region of rat pre-pro-C3 cdNA. The antisense ODN was synthesized with an Applied Biosystems DNA synthesizer (Model 394). A sense ODN complementary to the antisense ODN was synthesized as a control.

**Statistical Analysis**

Values are reported as mean±SEM. Statistical analysis was performed with Student t test for unpaired data, 2-way ANOVA, or Duncan multiple range test.

**Results**

**Systolic Blood Pressure**

Systolic blood pressure (SBP) of 3-week-old SHR and WKY rats was 113.3±2.3 mm Hg (n=6) and 110.8±3.4 mm Hg (n=6), respectively. There was no difference in SBP between SHR and WKY rats, and the SBP in SHR was within normal limits.

**Microarray Analysis**

Levels of transcripts that showed differential expression between aortic mid-layer smooth muscle from 3-week-old SHR and WKY rats by U34 GeneChip analysis are listed in the Table. Levels of 66 (5%) transcripts were significantly different between aortic mid-layer smooth muscle from 3-week-old SHR and WKY rats. Of these, 13 (1%) transcripts were expressed at significantly higher levels in smooth muscle from SHR than in that from WKY rats (Table), and levels of 52 (4%) were significantly higher in WKY rats (data not shown). Six (0.6%) transcripts, SDNT, EGF precursor, EEF2, OB-Rb, CALM, and pre-pro-C3, were detected only in aortic mid-layer smooth muscle from SHR. The U34 GeneChip allows assessment of C4, C8, and C9; however, there
Expression of C3 Protein in Cultured VSMCs

Expression of C3 protein in cultured VSMCs from SHR and WKY rats with or without interferon-γ (IFN-γ) treatment is shown in Figure 2. C3 protein was not expressed in VSMCs from WKY rats regardless of IFN-γ treatment, whereas C3 protein was expressed in VSMCs from SHR, and the level of expression was increased significantly (P < 0.05) by treatment with 10 ng/mL IFN-γ.

Effects of Exogenous C3 on Phenotype and Growth of VSMCs

The effects of exogenous C3 on phenotypic marker mRNAs and DNA synthesis by VSMCs from SHR and WKY rats are shown in Figure 3. Levels of SM22α mRNA were significantly lower (P < 0.05) and those of osteopontin and matrix Gla mRNAs were significantly higher (P < 0.05) in VSMCs from SHR than those in VSMCs from WKY rats. Basal DNA synthesis was significantly higher (P < 0.05) in VSMCs from SHR than that in VSMCs from WKY rats. These data suggest that the synthetic phenotype is present and that proliferation is faster in VSMCs from SHR than in cells from WKY rats. Exogenous C3 (0.1 μmol/L) significantly decreased (P < 0.05) the level of SM22α mRNA in VSMCs from both rat strains (Figure 3A). Exogenous C3 significantly increased (P < 0.01) expression of osteopontin (Figure 3B) and matrix Gla (Figure 3C) mRNAs in VSMCs from both rat strains. Exogenous C3 significantly increased (P < 0.05) DNA synthesis in VSMCs from both rat strains in a dose-dependent manner (Figure 3D). These data suggest that exogenous C3 can stimulate VSMCs to switch to the synthetic phenotype and stimulates growth of VSMCs.

Effects of Antisense ODN to Pre-Pro-C3 on Expression C3 Protein in VSMCs

To evaluate the contribution of endogenous C3 to the phenotype and growth of VSMCs from SHR, we designed and synthesized an antisense ODN to pre-pro-C3. The effects of the antisense and sense ODNs to pre-pro-C3 on expression of C3 protein in VSMCs from SHR are shown in Figure 1 (available online at http://www.hypertensionaha.org). Antisense ODN to pre-pro-C3 significantly decreased (P < 0.01) levels of C3 protein in a dose-dependent manner, whereas sense ODN had no effect on expression of C3 protein, indicating that the antisense ODN to pre-pro-C3 inhibited expression of C3 protein.

Effects of Antisense ODN to Pre-Pro-C3 on Growth of VSMCs

The effects of antisense and sense ODNs to pre-pro-C3 on growth of VSMCs from SHR and WKY rats are shown in Figure 4A. Doses of 0.1 and 1.0 μmol/L antisense ODN to pre-pro-C3 significantly inhibited (P < 0.01) basal DNA synthesis in VSMCs from SHR in comparison to that with sense ODN. In contrast, 0.1 μmol/L antisense ODN to pre-pro-C3 significantly increased (P < 0.05) DNA synthesis in VSMCs from WKY rats.

The effects of antisense and sense ODNs to pre-pro-C3 on proliferation of VSMCs from SHR and WKY rats are shown in Figure 4B. Proliferation was greater in VSMCs from SHR than in cells from WKY rats. Antisense ODN to pre-pro-C3 significantly (P < 0.01) suppressed proliferation of VSMCs from SHR cultured in the presence of 5% calf serum in a dose-dependent manner. Sense ODN did not affect proliferation of VSMC from WKY rats. Neither antisense nor sense
ODN to pre-pro-C3 affected proliferation of VSMCs from WKY rats.

**Effects of Antisense ODN to Pre-Pro-C3 on Expression of Phenotypic Marker mRNAs in VSMCs from SHR**

Doses of 0.01 and 0.1 μmol/L antisense ODN to pre-pro-C3 significantly increased ($P<0.01$) levels of SM22α mRNA in VSMCs from SHR, whereas 0.1 μmol/L antisense ODN to pre-pro-C3 significantly decreased ($P<0.01$) levels of osteopontin and matrix Gla mRNAs in VSMCs from SHR (Figure 5).

**Effects of Antisense ODN to Pre-Pro-C3 on Expression of Growth Factor mRNAs in VSMCs from SHR**

Antisense ODN to pre-pro-C3 significantly decreased ($P<0.01$) abundance of PDGF-A chain, TGF-β1, and bFGF mRNAs in a dose-dependent manner in VSMCs from SHR (Figure 6).

**Discussion**

In the present study, 6 transcripts, SDNT, EGF precursor, EEF2, OB-Rb, and CALM, and pre-pro-C3, were expressed only in aortic smooth muscle from SHR by microarray and RT-PCR analyses. Genes of SDNT, EGF precursor, EEF2, OB-Rb, and CALM were expressed in cultured VSMCs from both SHR and WKY rats. VSMCs in prolonged culture have a low-volume fraction of myofibrils and a high proportion of biosynthetic organelles. This is defined as the synthetic phenotype. It is considered that SDNT, EGF precursor, EEF2, OB-Rb, and CALM mRNAs were expressed in cultured VSMCs from WKY rats showing the synthetic phenotype, and the phenotype of the aortic smooth muscle from 3-week-old SHR appears to be the synthetic phenotype in vivo even in the prehypertensive stage.

Interestingly, pre-pro-C3 mRNA and C3 protein were expressed only in cultured VSMCs from SHR. It is possible that expression of C3 in VSMCs from SHR is caused by genetic alterations. Complement molecules are activated by complement activation systems, such as the classical activation pathway, the alternative activation pathway, and the mannose-binding lectin activation pathway. The microarray chip used in the present study (GeneChip U34) also allows assessment expression of C4, C8, and C9 mRNAs. There were no differences in levels of mRNA for C4, C8, and C9 in aortic smooth muscle from SHR and WKY rats, suggesting...
that the expression of C3 only in VSMCs from SHR is independent of the complement generating pathways.

C3 is the most abundant protein in the complement system and is also essential for the critical steps of the complement cascade, such as opsonization, generation of anaphylatoxins, and formation of the membrane attack complex. As extrahepatic expression of complement molecules, macrophages, fibroblasts, epithelial cells, endothelial cells, astrocytes, adipocytes, and myoepithelial cells have been reported as potential sources of complement molecules in tissues. It has been reported that VSMCs produce C3, C4, and C5. Ueda et al showed that C3 is produced by human aortic SMCs but not by a human SMC line or VSMCs from human umbilical cord vein. Thus C3 production differs between SMC lines. Production of C3 is regulated not only by immune complexes but also by several cytokines. Interleukin (IL)-1 and other proinflammatory cytokines, such as IFN-γ and IL-6, stimulate synthesis of C3 in several cell types. In the present experiments, expression of C3 was increased with IFN-γ in VSMCs from SHR, but these cytokines did not induce significant changes in C3 production in VSMCs from WKY rats.

C3 has roles in immune defenses but has also been reported to have several other biological functions. C3 is synthesized by embryonic cells, suggesting that this protein may participate in cell differentiation and proliferation. Moreover, C3 enhances growth of cancer cells. C3 contributes to contraction of VSMCs directly or indirectly through the activation of basophils and activates MAP kinase in endothelial cells. Thus, C3 appears to have direct effects on function and growth of VSMCs.

C3 is known to be increased in atherosclerotic lesions and to contribute to development of atherosclerosis in vivo. C3 produced by endothelial cells, monocyte/macrophages, and fibroblasts induces atherosclerosis via activation of several cytokines. However, C3 appears to induce the atherosclerosis as one of inflammations by stimulation of several cytokines in vivo. Therefore, we investigated the direct effects of C3 on the phenotype and growth of VSMCs from SHR.

SM22α is expressed preferentially in differentiated VSMCs, whereas osteopontin and matrix Gla are expressed strongly in dedifferentiated VSMCs. The present experiments, exogenous C3 significantly increased expression of SM22α mRNA and significantly increased expression of osteopontin and matrix Gla mRNAs in VSMCs from both rat strains. In addition, exogenous C3 significantly increased DNA synthesis in VSMCs from both rat strains. To our knowledge, this is the first report that C3 plays a direct role in stimulating VSMCs to change to the synthetic phenotype and that C3 stimulates growth of VSMCs.

We have shown that SHR-derived VSMCs possess the synthetic phenotype and have exaggerated growth in comparison with cells from WKY rats. Antisense ODN to pre-pro-C3 increased expression of SM22α mRNA and decreased expression of osteopontin and matrix Gla mRNAs. These data indicate that C3 contributes directly to the synthetic phenotype of VSMCs.
type of VSMCs from SHR. Moreover, antisense ODN to pre-pro-C3 reduced the higher basal DNA synthesis and proliferation observed in VSMCs from SHR and significantly inhibited expression of PDGF A-chain, TGF-β1, and bFGF mRNAs in these cells.

We have shown that levels of Ang II−generating enzymes cathepsin D and angiotensin-converting enzyme are increased with the synthetic phenotype.7,8 We recently demonstrated cathepsin D and angiotensin-converting enzyme are increased mRNAs in these cells.

Perspectives
C3 is produced only by VSMCs from SHR, and not by cells from WKY rats. C3 may be the protein that underlies the synthetic phenotype and exaggerated growth of VSMC from SHR. C3 will be a new target for therapy for hypertension.

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