Cardiac Ankyrin Repeat Protein (CARP) Expression in Human and Murine Atherosclerotic Lesions

Activin Induces Carp in Smooth Muscle Cells

Vivian de Waard, Tanja A.E. van Achterberg, Nicholas J. Beauchamp, Hans Pannekoek, Carlie J.M. de Vries

Objective—Cardiac ankyrin repeat protein (CARP) is a transcription factor–related protein that has been studied most extensively in the heart. In the present study, we investigated the expression and the potential function of CARP in human and murine atherosclerosis.

Methods and Results—CARP expression was observed by in situ hybridization in endothelial cells lining human atherosclerotic plaques, whereas lesion macrophages were devoid of CARP. Furthermore, we established that CARP mRNA and smooth muscle (SM) α-actin antigen both colocalized in a subset of intimal smooth muscle cells (SMCs), whereas no CARP mRNA was encountered in quiescent SMCs in the media. The CARP mRNA—expressing intimal SMCs were distinct from intimal SMCs that synthesized the activation marker osteopontin or proliferating cell nuclear antigen. In addition, we showed that activin A, a member of the TGFβ superfamily that prevents SMC-rich lesion formation, induced CARP mRNA expression in cultured SMCs.

Conclusions—Based on our data and the knowledge that CARP reduces the proliferation of cultured SMCs, we propose that CARP is involved in inhibition of vascular lesion formation. (Arterioscler Thromb Vasc Biol. 2003;23:64-68.)

Key Words: cardiac ankyrin repeat protein ❘ endothelial cells ❘ smooth muscle cells ❘ activin A ❘ atherogenesis

Cardiac ankyrin repeat protein (CARP) was originally identified in vascular endothelial cells, cardiomyocytes, skeletal muscle and, more recently, in vascular smooth muscle cells (SMCs).1–8 The presence of nuclear localization signals and multiple ankyrin repeats indicates that CARP is a transcription factor–related protein that is involved in protein–protein interactions. Identification of the sequence of CARP mRNA and protein revealed mRNA decay elements in the 3′-untranslated region and a PEST-rich region in the protein.1–5 These structural elements are involved in rapid mRNA and protein degradation, which is a typical feature of early response genes.

Until now, the function of CARP has been studied most extensively in the heart. Increased CARP expression is found in hypertrophy of the heart under physiological conditions (cardiogenesis) or pathophysiological conditions.3–7 Even though there are conflicting data on modulation of cardiac gene expression by CARP, a detailed analysis revealed that CARP prevents the activation of the MLC-2v promoter by binding the transcription factor YB-1 and consequently inhibits the activity of YB-1.5 Regulation of CARP expression in the heart is at least in part directed by the transcription factors Nkx2.5 and GATA-4, which are known to cooperate in the regulation of various cardiac genes.6,9–11 In skeletal muscle, CARP expression is induced in subcompartments of the muscle during normal morphogenesis or after denervation.2 In addition, in a myogenic cell line, CARP expression was upregulated on differentiation of these cells.2 In summary, CARP has been proposed to play a role in heart and skeletal muscle (re)differentiation.

During atherosclerosis, endothelial cells and SMCs become activated and play a key role in intima formation. Activated endothelial cells attract inflammatory cells, and SMCs proliferate and migrate into the subendothelial space. A potential role for CARP in atherosclerosis is suggested from the knowledge that endothelial cells and SMCs synthesize CARP on stimulation. Specifically, in cultured endothelial cells, inflammatory cytokines, such as interleukin-1 and tumor necrosis factor-α, have been shown to increase CARP expression.1 In cultured SMCs, CARP expression is enhanced by transforming growth factor (TGF)-β via the Smad signal transduction pathway, involving a TGF-β–response element in the CARP promoter. Importantly, adenovirus-mediated overexpression of CARP results in decreased proliferation of SMCs.8 Furthermore, CARP mRNA synthesis increased in balloon-injured rat aorta, as determined by reverse-transcription polymerase chain reaction (RT-PCR). Collectively, these data suggest a role for CARP during atherogen-
esis. However, the cellular origin and exact localization of CARP expression in the (atherosclerotic) vessel wall has not been assessed. Therefore, we have investigated CARP expression in human and murine atherosclerotic lesions and demonstrate that only a subset of endothelial cells and SMCs express CARP. We have recently shown that activin A, a TGF-β family member, prevents the formation of SMC-rich vascular lesions by maintaining the contractile SMC phenotype. In the current study we show induction of CARP mRNA expression in cultured SMCs on stimulation with activin A and we hypothesize a role for CARP downstream of activin A in preventing lesion formation.

**Methods**

**Human and Murine Tissue Samples**

Human vascular tissue of atherosclerotic lesions was acquired during vascular surgery. Informed consent was obtained from patients according to protocols of the Medical Ethical Committee of the Academic Medical Center of Amsterdam. Tissue samples were fixed within 15 minutes after resection in 3.8% (v/v) formaldehyde in PBS and were subsequently paraffin-embedded.

Murine vascular tissues were obtained from APOE*3-Leiden mice that were fed a Western-type (ie, high-fat/cholesterol) diet, resulting in the formation of macrophage-derived foam cell–rich lesions that contained limited numbers of intimal SMCs, as has been described by van Vlijmen et al. After 16 to 24 weeks of this Western-type diet, the mice were sacrificed, and hearts and aortas were harvested. Sections were taken at the aortic valves. At this time point, blood cholesterol levels of the APOE*3-Leiden mice were 38.5 ± 7.5 mmol/L, in contrast to 6.9 ± 0.2 mmol/L in control mice. In addition, murine lesions were obtained from mice of a C57Bl/6 genetic background that underwent carotid artery ligation. The carotid artery ligation model has been developed by Kumar and Lindner and results in the induction of SMC-rich lesions containing hardly any macrophages in the intima. Murine tissues were fixated by perfusion fixation with 4% (v/v) paraformaldehyde in PBS and were paraffin-embedded after resection. The sections (5 μm) were mounted on SuperFrost Plus glass slides.

**Immunohistochemistry**

Antibody 1A4 (DAKO) recognizes human and murine SM α-actin and was used to detect SMCs. HAM56 (DAKO) recognizes human macrophages, and PC10 detects proliferating cell nuclear antigen (PCNA) (DAKO). Immunohistochemistry was performed on sections that were rehydrated and blocked with 10% (v/v) preimmune goat serum (DAKO). Immunohistochemical protocol was continued. After visualization of the immunohistochemical protocol. Thereafter, antibodies were applied, and the immunohistochemical protocol was continued. After visualization of peroxidase activity, the sections were covered with nuclear research horseradish peroxidase conjugates (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl), followed by incubations with biotinylated secondary antibodies that were dehydrated and blocked with 10% (v/v) preimmune goat serum (DAKO). Subsequently, the sections were developed, these double-stained sections were counterstained with hematoxylin. In all in situ hybridization experiments, for each specimen 3 to 6 consecutive sections were applied.

**Cell Culture**

SMCs were obtained from human saphenous vein or iliac artery explant cultures and maintained at 37°C in a humidified 5% CO2 chamber in 40% (v/v) Medium-199 with L-glutamine/L-amino acids, 40% (v/v) RPMI 1640 HEPES-buffered/L-glutamine, and 20% (v/v) human serum supplemented with penicillin and streptomycin (GIBCO BRL). Cultured SMCs were characterized by immunofluorescence with the anti-SM α-actin 1A4 antibody. The SMCs show uniform fibroblastic staining. From five different cell cultures were made quiescent by incubation overnight in serum-free medium, supplemented with insulin (5 μg/mL), transferrin (5 μg/mL), selenite (5 ng/mL), GIBCO-BRL, and vitamin C (0.2 mmol/L). Subsequently, 100 ng/mL recombinant human activin A and 10 ng/mL TGFβ (Roche Diagnostics) was added to the culture medium during 0, 2, 8, 24, and 48 hours of the venous SMCs. The arterial SMCs were stimulated with various concentrations activin A (0, 10, 50, and 100 ng/mL) for 6 hours. Purified, recombinant human activin A (lot #15365 to 36) was obtained from Dr. Pawson through the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Disease, and the US Department of Agriculture (Bethesda, Md).

**RNA Isolation and Northern Blotting**

Total RNA was isolated from cultured cells by using the TRizol™ method (GIBCO-BRL). Ten micrograms of total RNA per lane was subjected to electrophoresis on a formaldehyde-agarose gel and transferred to Hybond nylon membranes (Amersham). The blot was prehybridized at 42°C in formamide-containing buffers according to standard procedures. Probes were radiolabeled with [35S]-UTP (Amersham) applying the random primer labeling mix (GIBCO BRL). To detect human CARP mRNA, a cDNA probe of 807 bp (454- to 1261-bp fragment of GenBank No. X83703) was applied and for GAPDH a fragment of 65 bp (480 to 545 bp of GenBank No. M33197). SM α-actin and SM22α mRNA were detected with a radiolabeled oligonucleotide and a random hexamer-radiolabeled cDNA probe, respectively, as has been described previously.

**RT-PCR**

cDNA was generated by reverse transcription of RNA samples by using oligo dT primers and Superscript II (GIBCO-BRL). CARP and hypoxanthine guanine phosphoribosyltransferase (HPRT) sequences were amplified from the cDNA samples with Taq DNA polymerase (Amersham) and transcript specific primers sets (CARP: 5'-GGGG-CAACTCCAGGATTTCC-3' and 5'-GGCATGCTTACCAATGG-3' and HPRT: 5'-TTAATTTGGACAGGACTGAC-3' and 5'-CCGAGCTTGTCCCTAC-3'). A standard PCR program with 65°C as annealing temperature resulted after 35 cycles in CARP and HPRT-specific products that were analyzed by agarose gel electrophoresis.

**Results**

**CARP Expression in Human Atherosclerotic Lesions**

To substantiate a potential function of CARP in human atherogenesis, we determined the exact expression pattern of CARP mRNA by in situ hybridization in human atherosclerotic lesions derived from aorta or iliac artery. We assayed 16 specimens derived from individuals ranging in age from 38 to 65 years with lesions varying from type II to type V that were classified according to guidelines from the American Heart Association (Table). Of all lesions examined, only one

To combine in situ hybridization and immunohistochemistry, human sections were blocked with 10% (v/v) preimmune goat serum in Tris-buffered saline after the last washing step of the in situ hybridization protocol. Thereafter, antibodies were applied, and the immunohistochemical protocol was continued. After visualization of peroxidase activity, the sections were covered with nuclear research horseradish peroxidase conjugates (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl), followed by incubations with biotinylated secondary antibodies that were dehydrated and blocked with 10% (v/v) preimmune goat serum (DAKO). Subsequently, the sections were developed, these double-stained sections were counterstained with hematoxylin. In all in situ hybridization experiments, for each specimen 3 to 6 consecutive sections were applied.

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CARP Expression in Endothelial Cells and SMCs in Vascular Specimens

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Origin</th>
<th>Sex</th>
<th>Age</th>
<th>Class</th>
<th>Details on Lesion Characteristics</th>
<th>EC</th>
<th>SMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aorta</td>
<td>M</td>
<td>41</td>
<td>II</td>
<td>Scattered Mϕ in SMC-rich lesion</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Aorta</td>
<td>M</td>
<td>49</td>
<td>II</td>
<td>Scattered Mϕ in SMC-rich lesion</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Aorta</td>
<td>M</td>
<td>49</td>
<td>II</td>
<td>Scattered Mϕ in SMC-rich lesion</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Aorta</td>
<td>F</td>
<td>38</td>
<td>II</td>
<td>Scattered Mϕ in SMC-rich lesion</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Aorta</td>
<td>F</td>
<td>38</td>
<td>II</td>
<td>Mϕ foam cells in SMC-rich lesion</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Aorta</td>
<td>F</td>
<td>41</td>
<td>II</td>
<td>Scattered Mϕ in SMC-rich lesion</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Aorta</td>
<td>F</td>
<td>41</td>
<td>II</td>
<td>Large fibrous lesion with SMCs and few Mϕ foam cells</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Aorta</td>
<td>F</td>
<td>41</td>
<td>III</td>
<td>Mϕ foam cells, SMCs and small extracellular lipid pools</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Iliac artery</td>
<td>F</td>
<td>41</td>
<td>V</td>
<td>Fibrous a-cellular core large SMC-rich fibrous cap and Mϕ foam cells in shoulder</td>
<td>nd</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>Iliac artery</td>
<td>F</td>
<td>41</td>
<td>II</td>
<td>Scattered Mϕ in SMC-rich lesion</td>
<td>nd</td>
<td>++</td>
</tr>
<tr>
<td>11</td>
<td>Iliac artery</td>
<td>F</td>
<td>41</td>
<td>V</td>
<td>Fibrous a-cellular core with large SMC-rich fibrous cap and Mϕ foam cells in deeper layer</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>12</td>
<td>Iliac artery</td>
<td>M</td>
<td>65</td>
<td>II</td>
<td>Fatty streak, Mϕ foam cells and SMCs</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>13</td>
<td>Iliac artery</td>
<td>M</td>
<td>65</td>
<td>II</td>
<td>Mϕ foam cells and SMCs</td>
<td>+</td>
<td>+</td>
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<td>14</td>
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<td>F</td>
<td>56</td>
<td>II</td>
<td>Fatty streak, Mϕ foam cells and SMCs</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>15</td>
<td>Iliac artery</td>
<td>F</td>
<td>56</td>
<td>IV</td>
<td>SMCs, large extracellular lipid pools and extensive Mϕ infiltrate, small a-cellular core, thin SMC-rich fibrous cap</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>16</td>
<td>Iliac artery</td>
<td>F</td>
<td>56</td>
<td>V</td>
<td>SMCs, large necrotic core, extensive Mϕ infiltrates and thin SMC-rich fibrous cap</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Class indicates the stage of atherosclerosis as classified according to the guidelines issued by the American Heart Association.16

EC indicates endothelial cells expressing CARP mRNA as determined by in situ hybridization; SMC, SMCs expressing CARP mRNA; +, <10% CARP-positive ECs or SMCs in a lesion; +++, 10%–50% CARP-positive cells; ++++, >50% CARP-positive cells; --, no CARP positive cells; nd, no ECs present presumably due to loss during specimen preparation; Mϕ, macrophages.

Lesion 7 is shown in Figure I (please see http://atvb.ahajournals.org) and lesion 15 in Figure II (please see http://atvb.ahajournals.org) and Figure 1.

To further delineate the expression profile of CARP in atherogenesis, we analyzed CARP expression in murine diet–induced atherosclerotic lesions and in lesions formed in a restenosis model. In APOE*3-Leiden transgenic mice, a Western-type diet results in the formation of lesions containing lipid-laden macrophages. Interestingly, the occasional SMCs present in these lesions, which form a fibrous cap, do express CARP (Figure 2A and 2B). Clearly, CARP mRNA colocalizes with SM α-actin as shown in a consecutive section (Figure 2C). CARP is only expressed in intimal SMCs and was never observed in medial SMCs or macrophages in this murine model, which is similar to our observations with human atherosclerotic specimens. Determination of the number of CARP-positive SMCs at different time periods of Western-type diet revealed that CARP reaches optimal expression after 16 weeks of diet, which correlates with the increased presence of intimal SMCs in these lesions (see online Figure IIIA, which can be accessed at http://atvb.ahajournals.org).

To evaluate the expression of CARP in intimal SMCs in further detail, we studied its expression pattern in lesions generated in the murine carotid artery ligation model. In the SMC-rich lesions induced in this model, CARP mRNA expression is present in a subset of intimal SMCs, indicating that intimal SMCs do not form a homogenous population (Figure 2D). To correlate CARP expression with a SMC phenotype, we analyzed in consecutive sections the expression of osteopontin and PCNA, which are markers for proliferative SMCs.17,18 In murine lesions, osteopontin may be considered a relatively broad activation marker, whereas PCNA is only expressed during a short period of the cell
cycle. A typical lesion is presented (Figure 2D through 2F), showing that the expression of CARP (D) does neither colocalize with osteopontin (E) nor with PCNA (F), which may indicate that CARP expression is not associated with intimal SMC proliferation. Analysis of temporal and spatial expression of CARP revealed that after 1 week of ligation, most CARP-positive cells are present in the media (Figure IIIB). At this time point, hardly any intima has been formed. Yet, medial SMCs are activated, express osteopontin (data not shown), and have started to proliferate and migrate to initiate the formation of a lesion. After 2.5 and 4 weeks of ligation, an extensive intima is present containing a subpopulation of CARP-positive SMCs. Already at 2.5 weeks, the medial SMCs are quiescent again and accordingly show only an occasional CARP-expressing SMC.

Note that we do not observe CARP expressing endothelial cells in the murine lesions. This could be either the result of the low number of endothelial cells present in the murine lesions, a different timeframe of CARP expression in the mouse, or a species difference between mice and humans.

Effect of Activin A Stimulation on CARP Expression
CARP expression is induced in SMCs by TGF-β via the Smad signal transduction pathway, which is mediated by a TGF-β response element in the CARP promoter. Activin A is a member of the TGF-β superfamily, and its signaling is mediated through the same Smad proteins as TGF-β signaling. To assess a potential effect of activin A on CARP expression, we added purified activin A for 2 to 48 hours to cultured SMCs and performed Northern blotting analyses (Figure 3A). The intensity of the bands, as determined by phosphorimager analysis and after correction for the GAPDH control, was plotted in line graphs (See online Figure IV, which can be accessed at http://
Ankyrin repeats in \( \text{I}^{\text{H}} \) are protein modules that are well studied in the \( \text{I}^{\text{H}} \) vascular lesions. First, CARP contains 4 ankyrin repeats, which are protein modules that are well studied in the \( \text{I}^{\text{H}} \) family. Ankyrin repeats in \( \text{I}^{\text{H}} \) are functionally involved in binding and inhibition of the transcription factor nuclear factor \( \text{xB} \). In line with the inhibitory function of \( \text{I}^{\text{H}} \) and in analogy to CARP binding of transcription factor \( \text{YB}^{-1} \) in cardiomyocytes, CARP may also be a negative regulator of transcription factors in endothelial cells and SMCs. Second, in human and murine lesions we have shown that CARP-expressing SMCs are a different subset of intimal SMCs than the subset of SMCs that express PCNA or osteopontin. Because PCNA and osteopontin have been associated with activated SMCs that are proliferative and migratory, CARP expression seems to be associated with quiescent intimal SMCs. It should be noted that CARP is not present in healthy vessels, and for that reason this protein is not simply a marker for quiescent SMCs. This is further illustrated by the presence of CARP in the media of murine lesions after 1 week of carotid artery ligation and the absence of expression after 2.5 and 4 weeks, at which time the medial SMCs have become quiescent again. Consequently, CARP may be involved in the transition of activated SMCs into a resting phenotype. Third, TGF-\( \beta \) and activin A (this study) are potent inducers of CARP expression in SMCs. Particularly, activin A is known as an inhibitor of atherogenesis, which is demonstrated by prevention of lesion formation both in saphenous vein organ cultures and in the murine, loose-fitting cuff model. Activin A has been shown to promote the contractile phenotype of SMCs, as is illustrated by the induction of expression of SM-specific markers, such as SM \( \alpha \)-actin and SM22\( \alpha \) on stimulation of cultured SMCs (Figure 3A). Fourth, overexpression of CARP in SMCs resulted in the inhibition of DNA synthesis. Kanai et al have shown that CARP-mediated inhibitory effects on SMC proliferation correlate well with increased levels of cyclin-dependent kinase inhibitor p21 and reduction of phosphorylation of the retinoblastoma protein Rb, involved in growth arrest.

Based on these data, we propose that the transient expression of CARP in intimal SMCs may be involved in preventing vascular lesion formation.

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