Endogenous Expression of C-Reactive Protein Is Increased in Active (Ulcerated Noncomplicated) Human Carotid Artery Plaques

Jerzy Krupinski, MD, PhD; Marta Miguel Turu, Msc; Jose Martinez-Gonzalez, PhD; Ana Carvajal, Msc; Josep Oriol Juan-Babot; Elena Iborra, MD; Mark Slevin, PhD; Francisco Rubio, MD; Lina Badimon, PhD

Background and Purpose—There is growing evidence suggesting that C-reactive protein (CRP) is an effector molecule able to induce and promote atherothrombosis. The presence of CRP in atherosclerotic plaques may reflect local production or infiltration from circulating CRP increased in general inflammatory responses. Our aim was to analyze the presence of CRP in human advanced carotid artery plaques with differential anatomo-pathological characteristics and to assess local expression of CRP and other proinflammatory genes in these lesions.

Methods—Human carotid artery specimens from 38 patients undergoing scheduled endarterectomy were classified into 3 groups: ulcerated (noncomplicated) (UNC, n=19), fibrous (F, n=12) and ulcerated (complicated/hemorrhagic) plaques (UC, n=7). The presence of CRP was evaluated by immunohistochemistry, and plasma samples were screened for circulating high-sensitivity C-reactive protein. TaqMan Low-density Arrays were used for study of genes related to inflammation (CRP, interleukin-6, macrophage colony-stimulating factor-1, monocyte chemotactic protein-1, cyclooxygenase-2).

Results—CRP mRNA levels were predominantly detected in UNC-high risk plaques but not in UC (P<0.001). UNC also exhibit the highest expression levels of other genes involved in the inflammatory responses: cyclooxygenase-2 (P<0.005 versus F and versus UC), IL-6 (P<0.005 versus F and versus UC) and monocyte chemoattractant protein-1 (P<0.01 versus F and versus UC). Plaque CRP mRNA levels correlated with immunohistochemical findings but were independent of plasma high-sensitivity CRP. In UNC plaques endothelial cells and inflammatory cells were strongly positive for CRP around areas of newly formed microvessels.

Conclusions—In human high-risk carotid artery plaques (UNC) CRP expression reflects an active proinflammatory stage. Local synthesis of CRP could be involved in plaque neovascularization and increased risk of hemorrhagic transformation. (Stroke. 2006;37:1200-1204.)

Key Words: angiogenesis, carotid atherosclerosis, C-reactive protein

Advanced atherosclerosis is thought to be a consequence of chronic local and systemic inflammatory disease. There are a large number of reports in favor of this hypothesis. Chronic inflammation is directly related to altered coagulation and subsequently higher risk of symptomatic vascular disease. C-reactive protein (CRP) is a powerful marker of future vascular events. In addition, there is an association between intimal CRP deposition and the development of atherosclerotic plaque. In fact, recent evidence support a role for CRP as an effector molecule able to induce proatherothrombotic phenotype in the vessel wall. CRP is a chemoattractant for monocytes, upregulates adhesion molecules, and decreases NO release by endothelial cells. CRP induces plasminogen activator inhibitor type 1 expression and tissue factor synthesis by vascular smooth-muscle cell (VSMC) and endothelial cells and exhibits both proliferative and apoptotic properties on vascular cells. CRP increases matrix metalloproteinase-1 in monocytes in culture suggesting that it could promote matrix degradation and thus contribute to plaque vulnerability. Extra-hepatic synthesis of CRP has been reported in alveolar macrophages, neurons, lymphocytes, and in vascular cells. In atherosclerotic lesions, immunohistochemical analysis demonstrated colocalization of CRP with VSMC and macrophages. In these lesions CRP mRNA has been detected by real-time polymerase chain reaction (RT-PCR).
and in situ hybridization in both VSMC and macrophages.\textsuperscript{16} CRP is mainly present in atherosclerotic lesions with active disease.\textsuperscript{17} In cell cultures CRP is produced by both VSMC and endothelial cells exposed to inflammatory cytokines\textsuperscript{18} or macrophage-conditioned medium.\textsuperscript{19} These data suggest that CRP present in atherosclerotic plaques may reflect local production rather than infiltration from circulating CRP produced by general inflammatory responses.

Although CRP seems to be a powerful proatherogenic and prothrombotic molecule, its possible effect on plaque complication and risk of subsequent vascular events has not been studied in detail. Identification of genes associated with vascular inflammatory biomarkers may provide new insights into local genetic determinants of vascular inflammation and risk of symptomatic vascular disease.\textsuperscript{20–21} The aim of the present study was to examine the presence of CRP in human advanced carotid artery plaques and to assess local expression of CRP and other inflammatory genes in these lesions.

Materials and Methods

Patients and Blood Sample Collection

We included 38 patients undergoing carotid endarterectomy for symptomatic (transient ischemic attack, minor stroke) or asymptomatic stenosis >70% confirmed by MR angiography or conventional arteriography. Patients were screened for the presence of bilateral pathology (>50% contralateral stenosis) by EcoDoppler imaging. The presence of vascular risk factors was recorded and previous antihypertensive, statin and antiplatelet treatments were noted (Table 1). Patients’ anticoagulated were not included in the study. Plasma samples were collected after overnight fasting and immediately stored at 80°C for further processing. There was no significant difference in time delay in the scheduled surgery. High-sensitivity CRP (hsCRP) was tested in EDTA-plasma samples by means of particle enhanced immunonephelometry using BN Systems (Dade Behring). For quantitative assay of hsCRP, patients with history of acute arterial or venous thromboembolism, active infections or inflammatory conditions, renal failure, hepatic disease, neoplasms, recent trauma or surgery were excluded. Patients with hsCRP values above 10 mg/L were retested. All other standard hematological and biochemistry analysis were routinely performed at the hospital laboratory. The study was approved by the local ethical committee in accordance with institutional guidelines.

Carotid Specimens/Immunohistochemistry

Carotid specimens were excised by the vascular surgeon without damage to the luminal surface. Additionally, carotid specimens from postmortem material (vascular transplants) were used as controls for mRNA and immunohistochemistry. They were immediately rinsed in 0.9% saline and cut longitudinally into 2 portions. One was snap frozen in liquid nitrogen and stored at −80°C and the other was fixed for 24 hours in buffered formalin and later cryoprotected in 30% sucrose and frozen in optimal cutting temperature for histology. Plaque morphology was assessed immediately after surgery and later by histology of hematoxylin and eosin-stained sections.\textsuperscript{22} Plaques were classified as ulcerated (UC) that exhibited hemorrhagic transformation, or fibrous (F). For immunohistochemistry, 8 μm serial sections were cut with a cryostat. The peroxidase method was used for immunohistochemical staining (Vectastain kit; Vector). After blocking endogenous peroxidase, the sections were incubated with normal serum and then incubated at 4°C overnight with the primary monoclonal anti-CRP antibody (clone CRP-8; Sigma; 1:100). Afterward, sections were incubated for 1 hour with secondary antibody (1:200) at room temperature. The peroxidase reaction was visualized with 0.05% diaminobenzidine.\textsuperscript{22} Negative controls in which the primary antibody was replaced with PBS was used to test for nonspecific binding (data not included).

RNA Extraction and cDNA Synthesis

RNA was isolated from carotid plaques frozen immediately after surgery by using Tripure isolation Reagent (Roche Molecular Biochemicals) according to the manufacturer. Integrity and concentration of RNA was measured using a Bioanalyzer (Agilent) and then total RNA was converted to single stranded cDNA using High-Capacity cDNA Archive Kit according to the manufacturer (Applied Biosystems).

TaqMan Low-Density Array

The TaqMan Low-Density Array is a 96 TaqMan Gene Expression assay (Applied Biosystems) preconfigured in a 384-well format and spotted on a microfluidic card (2 replicates per assay).\textsuperscript{23} Each TaqMan Gene expression assay contains a forward and reverse primer for each of the chosen genes (β-glucuronidase [GUS], CRP, interleukin-6 [IL-6], 18S ribosomal, macrophage colony-stimulating factor-1 [MCSF-1], monocyte chemotactic protein-1 [MCP-1] and cyclooxygenase-2 [COX-2]) at a final concentration of 900 nM and a TaqMan MGB probe (6-FAM dye-labeled; Applied Biosystems), 250 nM final concentration. GUS and 18S RNA were incorporated in our customized TaqMan Low-Density Array as internal standards. They were chosen among 11 candidate control genes previously checked using the TaqMan Human Endogenous Control Plate. Data were normalized using GUS because of the consistency of its level from sample to sample. First, 50 μL of cDNA from each sample was combined with an equal volume of TaqMan Universal PCR Master Mix (Applied Biosystems), and was loaded into each of 8 ports on the TaqMan Low-Density Array card. The real-time RT-PCR amplifications were run on an ABI Prism 7900HT sequence Detection System (Applied Biosystems) with a TaqMan Low-Density Array Upgarde. Thermal cycling conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for 1 minute.

<table>
<thead>
<tr>
<th>TABLE 1.</th>
<th>Patients Clinical Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feature</td>
<td>UNC</td>
</tr>
<tr>
<td>n</td>
<td>19</td>
</tr>
<tr>
<td>Male/female</td>
<td>16/3</td>
</tr>
<tr>
<td>Age</td>
<td>74±6</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>16</td>
</tr>
<tr>
<td>Hypertension</td>
<td>13</td>
</tr>
<tr>
<td>Diabetes</td>
<td>8</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>10</td>
</tr>
<tr>
<td>Smoking</td>
<td>13</td>
</tr>
<tr>
<td>CAD</td>
<td>10</td>
</tr>
<tr>
<td>Peripheral vasculopathy</td>
<td>5</td>
</tr>
<tr>
<td>Hypoechogenic plaque</td>
<td>6</td>
</tr>
<tr>
<td>Bilateral carotid pathology*</td>
<td>3±†</td>
</tr>
<tr>
<td>RAS-b†</td>
<td>9</td>
</tr>
<tr>
<td>Antiplatelets</td>
<td>9</td>
</tr>
<tr>
<td>Statins</td>
<td>7</td>
</tr>
</tbody>
</table>

*CAD indicates coronary artery disease; RAS-b, renin-angiotensin system blockers; NS, not significant.

†Contralateral carotid stenosis >50%; †RAS-b: patients taking either angiotensin converting enzyme inhibitors or angiotensin type II receptor antagonists; †P significant UNC vs UC.
TABLE 2. Blood/ Serum Parameters of Studied Subjects

<table>
<thead>
<tr>
<th>Feature</th>
<th>UNC</th>
<th>F</th>
<th>UC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC, ×10^9/L</td>
<td>7970 ± 2191</td>
<td>6502 ± 2560</td>
<td>7772 ± 1829</td>
<td>NS</td>
</tr>
<tr>
<td>Fibrinogen, g/l</td>
<td>4.8 ± 1</td>
<td>4.1 ± 0.8</td>
<td>4.9 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>hsCRP, mg/l</td>
<td>3.1 ± 3</td>
<td>2.5 ± 1.4</td>
<td>6.6 ± 5.9</td>
<td>NS</td>
</tr>
<tr>
<td>ESR, mm/H</td>
<td>21.4 ± 17</td>
<td>20.3 ± 14</td>
<td>13.3 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.1 ± 1</td>
<td>5.1 ± 1</td>
<td>4.2 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>LDL, mmol/l</td>
<td>3.2 ± 1</td>
<td>3.4 ± 0.8</td>
<td>3.1 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>HDL, mmol/l</td>
<td>1.1 ± 0.3</td>
<td>1.0 ± 0.2</td>
<td>1.1 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>TG, mmol/l</td>
<td>2.4 ± 0.4</td>
<td>2.1 ± 0.6</td>
<td>2.2 ± 0.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

WBC indicates white blood cells; ESR, erythrocyte sedimentation rate in 1 hour; LDL, low-density cholesterol; HDL, high-density cholesterol; TG, triglycerides; NS, not significant.

Results

Patient’s Baseline Characteristics

Patient’s demographic characteristics are presented in Table 1. Patients specimens were divided into 3 groups based on carotid plaque anatomic-pathological characteristics: UNC, F or UC. There was no significant difference in studied groups in gender, symptomatic carotid disease, individual or combined risk factors, treatment, or coexistence of other vascular disease. Patients with UNC had predominantly unilateral carotid pathology, ie, contralateral stenosis <50% as compared with others (P=0.02). There was no significant difference in basic biochemical parameters between studied groups (Table 2).

Expression of Inflammatory Genes in Carotid Plaques

Table 3 summarizes the results for The TaqMan Low-Density Array in carotid plaques. CRP mRNA was predominantly expressed in UNC but not in UC (P=0.001; Table 3). UC plaques were end-stage plaques with low cellular content and high necrotic component (see histology for details). CRP mRNA levels in fibroproliferative plaques was much lower than in ulcerated (P=0.01; Table 3). The increased CRP expression in UNC plaques was not associated with any particular cardiovascular risk factor or treatment (Table 1). Further, blood/serum parameters, ie, leukocytes, fibrinogen, erythrocyte sedimentation rate, or cholesterol were comparable in all 3 groups (Table 2). CRP mRNA levels were independent of plasma hs-CRP (P=0.4; r=0.032). Thus, CRP mRNA levels were directly related to plaque ulceration representing the active state of the plaque at the time of the study (Table 3). Interestingly, ulcerated plaques had higher expression of other genes involved in inflammatory responses (COX-2, IL-6 and MCP-1) than the rest of the carotid plaques. No significant differences were observed in MCSF-1 expression.

Immunohistochemistry for CRP

Plaque CRP mRNA levels correlated with immunohistochemical findings. In the UNC plaques CRP immunostaining corresponded to areas of high cellular content with inflammatory cells (Figure, a). Furthermore, areas of newly formed microvessels were also strongly positive for CRP (Figure, b). UNC plaques had higher content of CRP-positive endothelial cells and inflammatory cells than other carotid plaques (Table 4). There was also notable immunostaining in VSMC within neointima (Figure, c). In the end-stage plaques with important intraplaque hemorrhage or necrosis (UC) staining was much weaker or absent. CRP immunostaining was weak or absent in control plaques, postmortem normal carotid artery and fibrous plaques.

Discussion

The main finding of this study was that ulcerated, potentially vulnerable carotid plaques, but not ulcerated-complicated carotid plaques are biochemically active plaques that overexpress CRP and other inflammatory markers. In these plaques CRP was mainly located in neointimal areas enriched in inflammatory cells and microvessels. Furthermore, CRP expression level in these plaques was independent of cardiovascular risk factors, treatments, or plasma CRP levels. These findings seem to be important as so far all advanced carotid plaques in patients scheduled for endarterectomy have been considered as end-stage, and plaque progression measured by ultrasound usually estimates the grade of carotid stenosis rather than the exact stage of plaque activity.20

CRP is frequently deposited in atherosclerotic lesions from human and animal models; however, its origin and the pathological significance of CRP in these lesions are not completely understood. In humans, there is little information on CRP expression in vascular tissues.16,17,24–26 CRP expression in the human vascular wall was first detected by Yasojima et al in the thickened intima of middle aortic lesions colocalizing with VSMC and macrophages. Recent studies have found CRP mRNA in coronary arteries,24 femoral,25 and carotid plaques.26 Jabs et al reported that CRP was expressed in active sites of middle coronary atherosclerotic lesions (type IV and V), but not in end-stage plaques, and identified VSMC as the predominant cell type expressing CRP. Recently Sattler et al described important expression levels of inflammatory markers, including nuclear factor κB

TABLE 3. Inflammatory Genes in Carotid Plaques

<table>
<thead>
<tr>
<th>Genes Studied</th>
<th>UNC</th>
<th>F</th>
<th>UC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>0.6±0.2</td>
<td>0.2±0.03</td>
<td>0.1±0.08</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>COX-2</td>
<td>0.6±0.5</td>
<td>0.1±0.06</td>
<td>0.09±0.09</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>IL-6</td>
<td>12.6±11</td>
<td>0.4±0.3</td>
<td>0.2±0.03</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>MCP-1</td>
<td>6.2±5</td>
<td>2.1±0.9</td>
<td>1.8±1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MCSF-1</td>
<td>0.6±0.3</td>
<td>0.5±0.2</td>
<td>0.4±0.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS indicates not significant.
and CRP, in cell-rich areas from carotid endarterectomy specimens, especially in plaque shoulder and microvessels. However, it is not clear from the literature whether in situ expressed inflammatory markers differ in anatomo-pathological distinct carotid plaques.

We show that not all of the end-stage carotid plaques undergoing scheduled endarterectomy are biochemically equal. In fact, we identified a subgroup of "active" plaques (Ulcerated, UNC) that exhibit the highest levels of expression of CRP and other inflammatory markers (IL-6, COX-2 and MCP-1). In these plaques CRP was mainly located in cell-rich areas, VSMT, newly formed blood vessels and inflammatory cells, in agreement with results published by Sattler et al. Sattler et al. found that combined treatment with renin-angiotensin system blockers and ASA (COX-2 pathway blocking agents) decreased the expression of inflammatory markers in carotid arterial plaques. In our study, all patients were on antiplatelets and there was no difference in treatment with renin-angiotensin system blockers (angiotensin-converting enzyme inhibitors or angiotensin II receptor antagonists) in each group. However, these treatment modalities did not alter inflammatory marker expressed and relation to the anatomo-pathological state of the plaque.

In summary, the role of CRP in atherosclerotic lesions may go beyond inflammatory modulation, and we hypothesize that it could be a key player regulating lesion development and remodeling and triggering plaque rupture. Recent studies concluded that elevated hsCRP is an indirect risk factor for ischemic stroke based on its expression during the development of carotid atherosclerosis. In this study local CRP mRNA did not correlate with circulating hsCRP, however, expression was associated with vulnerable atherosclerotic lesion. To our knowledge this is the first study showing that intraplaque CRP could be regarded as a biomarker of a particular subgroup of "active" end-stage carotid plaques. Although measurement of intraplaque CRP could be important in the identification of vulnerable patients, currently available techniques do not permit for easy in situ CRP determination. Follow-up studies are required to confirm that local inflammatory responses and plaque hemorrhagic transformation are followed by vascular complications in these patients. However, such an intense local inflammatory response in advanced, ulcerated noncomplicated plaques suggests that these patients should be screened with new imaging techniques before the development of symptomatic disease.

**Acknowledgments**

This work was supported by the following grants: Marie Curie Reintegration grant (ERG-011511) to J.K.; Proyecto Ictus, Spain;
References

Endogenous Expression of C-Reactive Protein Is Increased in Active (Ulcerated Noncomplicated) Human Carotid Artery Plaques
Jerzy Krupinski, Marta Miguel Turu, Jose Martinez-Gonzalez, Ana Carvajal, Josep Oriol Juan-Babot, Elena Iborra, Mark Slevin, Francisco Rubio and Lina Badimon

Stroke. published online April 6, 2006;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/early/2006/04/06/01.STR.0000217386.37107.be.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/