C-Peptide Colocalizes with Macrophages in Early Arteriosclerotic Lesions of Diabetic Subjects and Induces Monocyte Chemotaxis In Vitro

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Objective—Increased levels of C-peptide, a cleavage product of proinsulin, circulate in patients with insulin resistance and early type 2 diabetes, a high-risk population for the development of a diffuse and extensive pattern of arteriosclerosis. This study tested the hypothesis that C-peptide might participate in atherogenesis in these patients.

Method and Results—We demonstrate significantly higher intimal C-peptide deposition in thoracic artery specimens from young diabetic subjects compared with matched nondiabetic controls as determined by immunohistochemical staining. C-peptide colocalized with monocytes/macrophages in the arterial intima of artery specimen from diabetic subjects. In vitro, C-peptide stimulated monocyte chemotaxis in a concentration-dependent manner with a maximal 2.3±0.4-fold increase at 1 mmol/L C-peptide. Pertussis toxin, wortmannin, and LY294002 inhibited C-peptide–induced monocyte chemotaxis, suggesting the involvement of pertussis toxin-sensitive G-proteins as well as a phosphoinositide 3-kinase (PI3K)-dependent mechanism. In addition, C-peptide treatment activated PI3K in human monocytes, as demonstrated by PI3K activity assays.

Conclusion—C-peptide accumulated in the vessel wall in early atherogenesis in diabetic subjects and may promote monocyte migration into developing lesions. These data support the hypothesis that C-peptide may play an active role in atherogenesis in diabetic patients and suggest a new mechanism for accelerated arterial disease in diabetes. (Arterioscler Thromb Vasc Biol. 2004;24:540-545.)

Key Words: C-peptide • monocytes • diabetes • arteriosclerosis

Patients with metabolic syndrome or frank type 2 diabetes mellitus have a particular propensity to develop a diffuse and extensive arteriosclerosis. Associated metabolic disorders like dyslipidemia, hyperglycemia, and hypercoagulability likely contribute to atherogenesis in these patients, but it remains unclear why these patients develop diffuse arteriosclerotic lesions early. Due to peripheral insulin resistance, these patients typically have increased serum levels of C-peptide, a cleavage product of proinsulin, released into the bloodstream in amounts equimolar to those of insulin. Despite several reports suggesting that C-peptide might modulate the release of insulin or glucagon from rat pancreas, little evidence supports biological activity of C-peptide. However, recent data suggest that C-peptide binds to specific yet unidentified cell surface receptors, thereby stimulating various intracellular signaling processes. C-peptide can induce Na-K-ATPase activity in rat renal tubular segments and pancreatic islet cells through activation of a pertussis toxin-sensitive G-protein-coupled receptor. Other recent work suggests that C-peptide stimulates mitogen-activated protein kinase (MAPK) in Swiss 3T3 fibroblasts and lung capillary endothelial cells, a process requiring protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K) activation. However, the effect of C-peptide on human monocytes and its potential role in early atherogenesis remains unexplored.

Impaired endothelial vasodilator function characterizes both early atherogenesis and insulin resistance and type 2 diabetes. In this phase that precedes lesion formation, the endothelium also displays increased permeability, permitting penetration of plasma constituents into the vessel wall, and monocytes accumulate at sites of developing lesions. In the subendothelium, these cells then become macrophages, major effectors of inflammation during atherogenesis.

Because endothelial dysfunction with increased permeability occurs in patients with insulin resistance and early type 2 diabetes, a group with generally high C-peptide serum levels,
we hypothesized that C-peptide deposits in the vessel wall in early atherosogenesis and promotes monocyte chemotaxis, thus fostering their migration into the lesion.

**Methods**

**Study Subjects**

To examine the deposition of C-peptide in early atherosclerotic lesions, we used postmortem artery specimens from the multicenter cooperative project “Pathobiological Determinants of Atherosclerosis in Youth” (PDAY). Study subjects were persons 15 through 34 years of age who died of external causes (accidents, homicides, suicides) within 72 hours after injury and were autopsied within 48 hours after death in one of the cooperating medical examiners’ laboratories. A 3.5-mm standardized OCT frozen block (PDAY section #9) was obtained from the dorsal, lesion-prone area of the thoracic aorta and used for sectioning and immunohistochemical analysis.

Data on cardiovascular risk factors, as well as data on HbA1c, cholesterol, and HDL obtained from blood samples collected at autopsy, were also available from the study subjects. Glycated hemoglobin was measured by affinity column chromatography (Helena Laboratories) as described.15 Methods for measuring the other risk factors in PDAY subjects are described in previous publications.16–18

**Immunohistochemical Staining**

Serial Cryostat sections of thoracic aorta specimens were cut, air dried onto microscope slides, and fixed in acetone at −20°C for 5 minutes. Staining for C-peptide was performed with a rabbit anti-human C-peptide antibody (Linco Research, St. Charles, MI) as well as a guinea pig anti-human C-peptide antibody (Fitzgerald, Concord, MA). Macrophages were identified by staining with a mouse anti-human CD68 antibody (Dako, Glostrup, Denmark). Staining for insulin used mouse anti-human insulin antibodies (US Biological, Swampscott, MA). Sections were preincubated with PBS containing 0.3% hydrogen peroxidase activity and stained for 1 hour with PBS supplemented with 10% appropriate serum. Negative controls used type and class matched IgG at similar concentrations or anti-C-peptide antibodies preabsorbed with recombinant human C-peptide (Sigma, Cambridgeshire, UK), subsequently using these “blocked C-peptide antibodies” at similar concentrations as experimental conditions. Preabsorption with insulin or the unspecific SI001 peptide at similar concentrations served as an additional control.

Finally, sections were incubated with the respective biotinylated secondary antibody (Dako) followed by avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Antibody binding was visualized with 3-amino-9-ethyl carbazole (Vector Laboratories) or with True Blue Peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Sections were counterstained with Gill’s Hematoxylin (Kirkegaard & Perry Laboratories). Computer-assisted image analysis was used to quantify staining on sections using Image-Pro Plus software (MediaCybernetics, Silver Spring, MD). Positive staining for C-peptide or CD68 was analyzed as percent area in the intima of the artery specimens.

**Immunofluorescence Staining**

Immunofluorescence staining for C-peptide and macrophages used the same antibodies described above. Sections were preincubated with PBS containing 5% respective serum. Primary antibodies (1:50, anti-C-peptide; 1:25, anti-CD68) diluted in PBS containing 3% serum were added for 1 hour at room temperature. After washing with PBS, Alexa Fluor 488- and carboxymethylindocyanine 3 (Cy3; Dianova, Hamburg, Germany)-coupled goat anti-mouse or anti-rabbit IgG were added as secondary antibodies for 45 minutes. Images were recorded with a confocal laser-scanning microscope (Leica, Solms, Germany). To quantify C-peptide positivity of intimal monocytes/macrophages in sections from diabetic subjects, CD68-positive cells in each section were counted according to their green fluorescence (Alexa Fluor 488) and scored for the presence of C-peptide (Cy3) by analyzing the double fluorescence of CD68 and C-peptide.

**Monocyte Chemotaxis Assay**

Human monocytes were isolated from freshly drawn blood of healthy volunteers using serial Ficoll/Percoll gradient centrifugation, as described.19 Cells were cultured for 16 hours in RPMI-1640 media supplemented with 0.5% human serum to become quiescent after isolation. Purity of the cells was >95% as determined by flow cytometry analysis. Monocyte chemotaxis was assayed in a 48-well microchemotaxis chamber (Neuroprobe, Gaithersburg, MD) in serum-free media.20 Wells in the upper and lower chamber were separated by a polycarbonate membrane (pore size 5 μm; Costar). Freshly isolated monocytes at a density of 5 × 10^6/mL were incubated for 2.5 hours with recombinant C-peptide (Sigma), insulin, or MCP-1 before migrated cells on the bottom face of the filter were stained and counted under the light microscope. Cells were counted in 5 random high-power fields per well. Checkboard analysis was performed to differentiate chemotactic from chemokinetic activity. In some experiments, monocytes were pre-treated with pertussis toxin, Wortmannin, and LY294002 (both PI3K inhibitors).

**PI3K Assay**

Isolated monocytes were stimulated for 5 minutes before lysis in a buffer containing 20 mmol/L HEPES, pH 7.5, 10 mmol/L EGTA, 1% NP-40, 2.5 mmol/L MgCl2, 2 mmol/L sodium orthovanadate, 40 mmol/L β-glycerophosphate, 1 mmol/L DTT, 40 μg/mL PMSF, 10 μg/mL leupeptin, 2 μg/mL pepstatin A, and 2 μg/mL aprotinin. In some experiments, cells were pretreated (30 minutes) with Wortmannin or pertussis toxin before stimulation with C-peptide. PI3K was immunoprecipitated with anti-PI3K (Santa Cruz Biotechnology) in the presence of protein A/G agarose (Santa Cruz Biotechnology). The immunocomplex was washed 3 times. After washing with lysis buffer and lipid kinase buffer (20 mmol/L TRIS pH 7.4, 4 mmol/L MgCl2, 100 mmol/L NaCl), immunocomplexes were resuspended in 25 μL of 1× lipid kinase buffer and added to 32 μL of 3× lipid kinase buffer (60 mmol/L TRIS pH 7.4, 12 mmol/L MgCl2, 300 mmol/L NaCl), 12 μL phosphatidylinositol (10 mg/mL, Sigma), and 10 μL [γ-32P] ATP (10 μCi). After incubation for 30 minutes at 30°C, the reaction was stopped by addition of 150 μL of 1 mol/L HCl. The organic phase was separated by addition of 450 μL methanol/chloroform with the volume ratio of 4:1. The lipids in the organic phase were separated on TLC silica gel plate (Whatman). TLC plates were developed in methanol/chloroform/H2O/ammoniak with the volume ratio of 47:60:11:3. Radioactive PIP products were visualized by autoradiography.

**Statistical Analysis**

Normal distribution of data were tested using the Kolmogorov-Smirnov Test. Differences between diabetic (HbA1c ≥8%) and nondiabetic (HbA1c <8%) subjects were analyzed using Student’s t-test or using the rank sum test for skewed parameters. Skewed data were reported as median (interquartile range); all other data as mean±SEM. Correlation of C-peptide deposition with HbA1c levels was analyzed using Spearman rank correlation. Results of the experimental studies are reported as mean±SEM. Differences were analyzed by 1-way-ANOVA followed by the appropriate posthoc test. A probability value <0.05 was regarded as significant.

**Results**

**C-Peptide Deposits in Early Atherosclerotic Lesions in Diabetic Subjects**

To examine the deposition of C-peptide in early atherosclerotic lesions, we used postmortem thoracic artery specimens from the PDAY Study. Subjects with HbA1c levels ≥8% were classified as diabetic, as done previously in analyses of this study.21 Diabetic and nondiabetic subjects, from whom artery specimens were chosen, did not significantly differ in any of the characteristics available (age, race, postmortem index, cholesterol levels, HDL levels, smoking status).
HbA1c levels were 8.5±0.1% in the diabetic and 6.6±0.1% in the nondiabetic group (P<0.001; n=21 in both groups; Table). Intimal thickness did not significantly differ between the two groups (Table). Immunohistochemical analysis of thoracic aorta specimens demonstrated prominent C-peptide deposition in the subendothelium and intima of diabetic patients (Figure 1A and 1B). Some subjects also had C-peptide in the arterial media. Two different anti-human-C-peptide antibodies showed similar results. Staining of sections with an isomatched IgG at similar concentration or with anti-C-peptide antibodies preabsorbed with recombinant C-peptide showed no immunoreactivity, thus affirming the specificity of the detected signals (Figure 1C and 1D). Preabsorption with insulin as well as with a nonspecific peptide (S100-peptide) at similar concentrations did not inhibit C-peptide staining (data not shown). Arteries of nondiabetic subjects showed little or no C-peptide deposition (Figure 1E and 1F). The extent of C-peptide deposition in the intima of thoracic aorta specimens of diabetic subjects significantly exceeded that of nondiabetic subjects, as determined by computer-assisted image analysis (Figure 1, available online at http://atvb.ahajournals.org).

To examine the specificity of C-peptide deposition, we also performed immunohistochemical staining for insulin. None of the sections of diabetic or nondiabetic subjects exhibited insulin immunoreactivity (Figure IIa, available online at http://atvb.ahajournals.org), while adjacent sections stained positive for C-peptide (Figure IIC, available online at http://atvb.ahajournals.org). Frozen sections of pancreatic tissue positive for C-peptide (Figure IIC, available online at http://atvb.ahajournals.org), while adjacent sections stained positive for C-peptide in the arterial media. Two different anti-human-C-peptide antibodies showed similar results. Staining of sections with an isomatched IgG at similar concentration or with anti-C-peptide antibodies preabsorbed with recombinant C-peptide showed no immunoreactivity, thus affirming the specificity of the detected signals (Figure 1C and 1D). Preabsorption with insulin as well as with a nonspecific peptide (S100-peptide) at similar concentrations did not inhibit C-peptide staining (data not shown). Arteries of nondiabetic subjects showed little or no C-peptide deposition (Figure 1E and 1F). The extent of C-peptide deposition in the intima of thoracic aorta specimens of diabetic subjects significantly exceeded that of nondiabetic subjects, as determined by computer-assisted image analysis (Figure 1, available online at http://atvb.ahajournals.org).

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C-peptide Colocalizes with Monocytes/Macrophages in Thoracic Artery Specimens of Diabetic Subjects

Immunohistochemical staining of parallel sections (Figure 2A through 2D) as well as immunofluorescence staining (Figure 2E through 2G) demonstrated colocalization of C-peptide with CD68-positive monocytes/macrophages in the arterial intima. Despite intimal immunoreactive C-peptide in all diabetic subjects, monocyte/macrophage infiltration was absent in 5 of 21 subjects (23%). Quantitative image analysis of immunofluorescent images of sections from diabetic subjects demonstrated that 96% of all intimal monocytes/macrophages colocalized with C-peptide. These data suggest that C-peptide deposition may precede monocyte recruitment into the vessel wall.

C-Peptide Induces Monocyte Migration In Vitro

Evaluation of C-peptide’s effect on monocyte migration used isolated human monocytes in an in vitro chemotaxis assay. Stimulation of monocytes with C-peptide stimulated cell migration in a concentration-dependent manner with a maximal augmentation of 2.3±0.4 at 1 nmol/L (P<0.05 compared with unstimulated cells, n=18; Figure 3A). Heat-inactivation of C-peptide (1 nmol/L) abolished the migratory effect of C-peptide (P is not significant compared with unstimulated cells, n=15), rendering endotoxin contamination as an unlikely responsible mechanism. The extent of C-peptide–induced monocyte migration resembled the effect of the established monocyte chemokine MCP-1, which increased monocyte migration by 2.9±0.2 fold (P<0.05, n=10; Figure 3B). In contrast to C-peptide, insulin had no effect on monocyte migration (Figure 3B).

Testing of C-peptide’s chemotactic activity (versus chemokinesis) used a checkerboard analysis with serial dilutions of C-peptide above and below the filter. Monocyte migration depended on the presence of a C-peptide gradient between the upper and the lower face of the filter, suggesting that C-peptide induces directed migration of monocytes rather than mere chemokinesis (n=12; Figure 3C).

C-Peptide–Induced Monocyte Migration Involves Pertussis Toxin Sensitive G-Proteins and PI3K

To investigate further intracellular signaling pathways involved in C-peptide–induced monocyte chemotaxis, inhibition migration experiments were performed. Previous work with kidney cells has shown that C-peptide signaling involves pertussis toxin-sensitive G-proteins.9 Treatment of human monocytes with pertussis toxin (0.5 µg/mL) inhibited C-peptide–induced monocyte migration (Figure 4A), suggesting that pertussis toxin-sensitive G-proteins are involved. Because some G-protein coupled receptors activate PI3K, and given the involvement of PI3K on monocyte motility,22 we next examined the role of PI3K
activation in C-peptide–induced monocyte migration. Inhibition of PI3K by two different reagents, wortmannin (100 nmol/L) as well as LY294002 (500 nmol/L), significantly reduced C-peptide–induced monocyte migration (Figure 4A). Pertussis toxin, wortmannin, or LY294002 did not affect cell viability as examined by trypan blue staining (data not shown). These data suggest that C-peptide–induced monocyte migration involves pertussis toxin sensitive G-proteins as well as PI3K.

C-Peptide Activates PI3K in Human Monocytes
To demonstrate that C-peptide treatment activates PI3K in human monocytes, cells were stimulated with C-peptide (1 nmol/L) for 5 minutes before a PI3K activity assay was performed. C-peptide activated PI3K in human monocytes, while heat-inactivated C-peptide had no effect. As expected, wortmannin reduced C-peptide–induced PI3K activation. In addition, pertussis toxin also inhibited C-peptide–induced PI3K activity, suggesting the involvement of pertussis toxin-sensitive G-proteins upstream of PI3K (Figure 4B).
Discussion

The present study demonstrates increased C-peptide deposition in early arteriosclerotic lesions of diabetic subjects, as well as intimal colocalization of C-peptide with monocytes/macrophages in the vessel wall. In addition, this study reports chemotactic effects of C-peptide on monocytes in vitro. These data support the novel hypothesis that C-peptide may promote early atherogenesis in patients with type 2 diabetes mellitus by initiating or furnishing monocyte migration into developing lesions.

To compare C-peptide deposition in diabetic and nondiabetic subjects, we used thoracic aorta specimens from subjects of the PDAY Study. Diabetic patients were classified as having HbA1c levels ≥8% and compared with matched controls having an HbA1c <8%, as described. Most diabetic subjects have type 2 diabetes, but classification of subjects by their HbA1c levels cannot exclude that some of the patients might have type 1 diabetes, thus potentially having low C-peptide serum levels despite an elevated HbA1c. In addition, some of the subjects with low HbA1c levels might have been type 2 diabetic patients under antidiabetic treatment. Despite these potential confounders, C-peptide deposition significantly differed among the groups, suggesting that C-peptide insudates into the vessel wall.

C-peptide–induced monocyte migration involves pertussis toxin–sensitive G-proteins as well as PI3K. A, Pertussis toxin (PTX at 0.5 μg/mL), wortmannin (100 nmol/L), and LY294002 (500 nmol/L) reduce C-peptide–induced monocyte chemotaxis. Bars represent mean ± SEM expressed as percent of C-peptide-stimulated cells. *P < 0.05 compared with C-peptide-stimulated cells (n = 11). B, C-peptide activates PI3K in human monocytes. Human monocytes were treated with C-peptide (1 nmol/L) for 5 minutes before a PI3K activity assay was performed. Also shown are cells treated with C-peptide in the presence of wortmannin (Wortm at 100 nmol/L) or pertussis toxin (PTX at 0.5 μg/mL), as well as monocytes treated with heat-inactivated (HI) C-peptide. Specific dots are labeled with an arrow (PIP). Three independent experiments yielded similar results.

Figure 3. C-peptide induces monocyte migration in vitro. A, C-peptide concentration dependently induces migration of isolated human monocytes. Heat-inactivated C-peptide (HI) shown as control. Data are expressed as fold induction compared with unstimulated cells. Bars represent mean ± SEM (n = 18; *P < 0.05). B, The extent of C-peptide–induced monocyte migration is similar to the effect of the established monocyte chemokine MCP-1. Also shown is the effect of insulin on monocyte migration. Data are expressed as fold induction of unstimulated cells. Bars represent mean ± SEM (n = 5 for C-peptide and insulin; n = 10 for MCP-1; *P < 0.05 compared with unstimulated cells). C, C-peptide induces monocyte chemotaxis rather than chemokinesis. Checkerboard analysis revealed that monocyte migration depended on the presence of a C-peptide gradient across the filter, suggesting that C-peptide induces monocyte chemotaxis rather than chemokinesis. Data are expressed as fold induction compared with unstimulated cells. Bars represent mean of 12 independent experiments.
further projects to examine this correlation and to elucidate the kinetics of C-peptide deposition in the vessel wall. In the vasculature, C-peptide colocalizes with monocytes/macrophages. Because 23% of the sections from diabetic subjects demonstrated C-peptide immunoreactivity but lacked monocytes/macrophages, C-peptide deposition in the vessel wall might precede monocyte infiltration. In addition, given that 96% of the intimal macrophages colocalized with C-peptide, and given the chemotactic effects of C-peptide on human monocytes described here, C-peptide might deposit in the subendothelium of diabetic patients and subsequently attract monocytes to migrate into the vessel wall.

The effect of C-peptide, inducing monocyte migration by 2.0 to 2.3 fold, is modest in vitro, but might be important and physiologically relevant over years. The C-peptide concentrations used in our migration experiments fall within the range of normal C-peptide serum concentrations (0.5 to 1.5 nmol/L), and because C-peptide–induced monocyte migration would depend on a concentration gradient between blood and subendothelial space, the concentrations employed might resemble the in vivo situation.

Previous work has shown that C-peptide binds to a cell surface receptor, but this receptor remains unidentified. Work in renal cells implies a pertussis toxin-sensitive G-protein–coupled receptor in mediating C-peptide’s effects, and our data, showing an inhibitory effect of pertussis toxin on C-peptide–induced monocyte migration, suggest that similar pathways are involved here. Downstream of these pertussis toxin-sensitive G-proteins, C-peptide stimulation results in an increase in PI3K activity. PI3K is known to contribute to monocyte migration induced by other stimuli, such as colony-stimulating factor-1 (CSF-1), or as growth hormone. Moreover, previous studies have shown that C-peptide activates PI3K in Swiss 3T3 fibroblasts and lung endothelial cells, suggesting that this signaling pathway might mediate C-peptide’s effects in different cell types. Further work should address the identity of a putative C-peptide receptor in human monocytes and the downstream intracellular molecules involved in signal transduction.

The present study suggests an active role for C-peptide in the development of atherosclerotic lesions in patients with type 2 diabetes. By accumulation in the vessel wall and stimulation of chemotaxis as shown here, C-peptide could aid recruitment of monocytes into developing atheroma and thus contribute to a crucial step in plaque formation. Our data raise a novel hypothesis to explain the diffuse and extensive pattern of atherosclerosis in patients with type 2 diabetes mellitus. These initial observations should stimulate further studies to elucidate whether C-peptide might contribute to accelerated lesion development in diabetic subjects and to examine further the intracellular signaling mechanisms of C-peptide–induced monocyte migration.

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