Detection of Monocyte Chemoattractant Protein-1 Receptor Expression in Experimental Atherosclerotic Lesions
An Autoradiographic Study
Katsuichi Ohtsuki, MD; Motoya Hayase, MD; Koichi Akashi, MD; Susan Kopiwoda, MS; H. William Strauss, MD

Background—Monocytes, a common component of atheroma, are attracted to the lesion site in response to chemotactic signals, particularly expression of monocyte chemoattractant peptide 1 (MCP-1). This study assessed the feasibility of using radiolabeled MCP-1 to identify monocytes and macrophages that have localized at sites of experimental arterial lesions.

Methods and Results—The biodistribution of radiolabeled MCP-1 was determined in normal mice, and localization in experimental atheroma was determined in cholesterol-fed rabbits 4 weeks after arterial injury of the iliac artery (9 rabbits) and the abdominal aorta (1 rabbit). Vessels were harvested and autoradiographed after intravenous administration of 125I-labeled MCP-1 and Evans blue dye. The arteries were evaluated histologically by hematoxylin and eosin staining and immune staining with a monoclonal antibody specific for rabbit macrophages (RAM-11). 125I-MCP-1 has a blood clearance half-time of ≈ 10 minutes and circulates in association with cells. The liver, lungs, and kidneys had the highest concentration of 125I-MCP-1 at 5 and 30 minutes after tracer administration. Autoradiograms revealed accumulation of 125I-MCP-1 in the damaged artery wall, with an average ratio of lesion to normal vessel of 6:1 (maximum 45:1). The accumulation of 125I-MCP-1 in the reendothelialized (plaque formation) areas was greater than in the deendothelialized (Evans blue–positive) areas (6.55±2.26 versus 4.34±1.43 counts/pixel, P<0.05). The uptake of 125I-MCP-1 correlated with the number of macrophages per unit area (r=0.85, P<0.0001).

Conclusions—Radiolabeled MCP-1 may be a useful tracer for imaging monocyte/macrophage-rich experimental atherosclerotic lesions. (Circulation. 2001;104:203-208.)

Key Words: monocyte chemoattractant proteins ■ atherosclerosis ■ macrophages ■ autoradiography

Coronary atherosclerosis ranges from lesions that are likely to rupture, vulnerable plaque, to stable, calcified lesions. Vulnerable plaque is characterized by a thin cap, a necrotic core (associated with lakes of lipid), and a marked cellular infiltration containing monocytes, macrophages, and foam cells. Monocytes are attracted to sites of arterial injury by several substances, such as monocyte chemoattractant protein-1 (MCP-1), interleukin 1, tumor necrosis factor-α, and interferon-γ, that are produced by cells at sites of inflammation. Monocytes migrating to the site of cytokine production enter the lesion, transform into macrophages, and remain in the lesion. In arterial lesions, macrophages may progress to foam cells by accumulating lipoprotein cholesterol through scavenger receptor–mediated endocytosis.

MCP-1, a monomeric polypeptide with a weight of 9 to 15 kDa, is produced by endothelial cells, smooth muscle cells, and monocytes/macrophages. The peptide is recognized by CCR-2 receptors expressed on monocytes. MCP-1 is a potent monocyte chemoattractant and is largely responsible for the recruitment of monocytes/macrophages to the vessel wall in the process of atherogenesis. We hypothesized that lesions with high concentrations of macrophages could be identified by labeling of cells that were already attracted to the site of inflammation. Because activated macrophages express a high concentration of chemotactic receptors on their surface, we selected radiolabeled MCP-1 (125I-MCP-1) to test this hypothesis. MCP-1 is available as a research peptide from a number of vendors, including Peprotech. The material can also be obtained as iodinated MCP-1 from the New England Nuclear Co.)
Methods

Three sets of experiments were performed: First, the biodistribution of radioiodinated MCP-1 was determined in normal mice. Second, the time course of blood clearance and the partition of activity between cells and plasma was determined in a rabbit after intravenous injection of MCP-1. Third, localization of radiolabeled MCP-1 was determined by autoradiography in rabbits fed an atherogenic diet who had sustained a balloon deendothelialization injury.

Mouse Biodistribution

One microcurie (37 kBq) of \(^{125}\text{I}\)-labeled recombinant human MCP-1 (New England Nuclear Co, \(\sim\)1 pmol [13 ng]) of peptide was injected intravenously into 20-g male BALB/c mice. Mice were killed at 5, 30, 60, and 180 minutes after administration (n = 6 per time point). Samples of blood, thymus, lungs, heart, thyroid, lymph node, abdominal aorta, vena cava, liver, spleen, kidneys, and stomach were harvested, rinsed in saline, weighed, and counted in a well-type scintillation counter (Packard Cobra II, baseline 15 keV, upper level 75 keV). The results were expressed as percent injected dose per gram tissue (% ID/g) and percent injected dose per organ (% ID/organ).

Blood Clearance and Plasma/Whole Blood Partition of \(^{125}\text{I}\)-MCP-1 in a Rabbit

The blood clearance and plasma/cell partition of MCP-1 was determined to define an optimal time to sample the vessels in the lipid-fed rabbits. Thirty microcuries of \(^{125}\text{I}\)-labeled MCP-1 was injected intravenously into an anesthetized rabbit, and blood samples (0.2 mL) were collected from the contralateral ear over a period of 3 hours. Radioactivity of whole blood and plasma was measured with a gamma-well counter and expressed as percent injected dose per gram.

Experimental Atherosclerotic Lesions

Nine male New Zealand White rabbits weighing 3.0 to 3.4 kg (Charles River Breeding Laboratories, Wilmington, Mass) were maintained on a high-cholesterol diet (1% cholesterol, Dyets Inc) for 5 weeks. After 1 week on the diet, 1 iliac artery of all 9 rabbits and the aorta of 1 (to determine whether the pattern of localization would be similar in vessels of different caliber) were injured. 14 Animals were anesthetized with ketamine (3 mg/kg) and xylazine (5 mg/kg), a 5F sheath was inserted under fluoroscopy into the right carotid artery, and a guidewire was passed to the iliac artery. An angioplasty balloon (3 mm; Advanced Cardiovascular Systems) was advanced into the iliac artery, inflated to 8 atm, and pulled back to the abdominal aorta 3 times. In addition to the iliac artery, the aorta of 1 rabbit was injured in a similar fashion, with a balloon inflated to 10 atm in the proximal iliac vessel and pulled back to the diaphragm. The carotid artery was ligated, and the wound was closed. The experimental protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University.

After 4 additional weeks of diet, 45 \(\mu\text{Ci}\) (1.67 MBq) of \(^{125}\text{I}\)-labeled MCP-1 was injected intravenously, followed 2 hours later by 4 mL of a 0.5% solution of Evans blue dye (Sigma) to stain the deendothelialized aorta. One hour later (3 hours after \(^{125}\text{I}\)-MCP-1), the animals were killed with an overdose of sodium pentobarbital. The distal aorta and both iliac arteries were stained the deendothelialized aorta. One hour later (3 hours after injection of \(^{125}\text{I}\)-MCP-1), the animals were killed with an overdose of sodium pentobarbital. Sections were scanned with \(\times4\) magnification and digitized with the Image Analyst Program (Automatix). The external and internal elastic lamina and lumen/intima border were identified, and an intima/media ratio was calculated. The intima was defined as the vessel layer between the internal elastic lamina and the intima/lumen border, and the media was defined as the area between the internal and external elastic laminae. Three regions were measured for each sample.

Immunohistochemistry

Tissue slices adjacent to those used for hematoxylin and eosin staining were stained with an antibody specific for rabbit macrophage (RAM-11, Dako). Sections were incubated with primary antibody, anti-rabbit IgG secondary antibody (biotin conjugate), and avidin peroxidase for 20 minutes. Peroxidase was visualized with chromagen (Zymed Laboratories Inc). Three cross sections were immunostained for each tissue. RAM-11-positive cells in the intima and media were counted under \(\times100\) magnification in 4 to 6 regions and expressed as the number per 0.25 mm².

Autoradiography

The vessels were covered with 1 layer of plastic (Saran) wrap, placed on x-ray film (Kodak Diagnostic Film Min-R), and stored for 10 weeks in a Kodak Min-R2 cassette. The autoradiographs were developed, scanned (300 DPI, 256 grey shades), and quantified by use of the public domain NIH Image program. Regions of interest (4 to 6 mm²) were set on the denuded, plaque, and uninjured areas and background (6 regions each) as for morphometric and immunohistochemical analysis. The relative density of the injured vessel area and uninjured area (counts/pixel) was obtained after background correction. The accumulation ratio of tracer in each injured area was defined as the ratio of its regional density to the mean density of 4 uninjured regions.

Statistical Analysis

Results were expressed as mean±SD. Comparisons between 2 groups were made with a 2-tailed Student’s t test for paired data. Variables among 3 groups were compared by ANOVA with Schef- fe’s post hoc test. The accumulation ratio of \(^{125}\text{I}\)-MCP-1 was

<table>
<thead>
<tr>
<th>Tissue Sample</th>
<th>5 min (6)</th>
<th>30 min (6)</th>
<th>60 min (6)</th>
<th>180 min (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>37.80±3.68</td>
<td>17.27±1.12</td>
<td>9.70±3.26</td>
<td>2.86±0.53</td>
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<td>Lungs</td>
<td>54.50±7.66</td>
<td>63.35±9.11</td>
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<td>3.82±4.41</td>
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<td>1.66±0.96</td>
<td>0.36±0.08</td>
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<td>Artery</td>
<td>10.77±2.16</td>
<td>5.77±0.61</td>
<td>4.71±1.73</td>
<td>0.96±0.23</td>
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<tr>
<td>Vein</td>
<td>10.12±3.76</td>
<td>7.20±0.38</td>
<td>4.74±1.55</td>
<td>1.21±0.45</td>
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<td>Thyroid</td>
<td>5.20±1.00</td>
<td>4.93±0.44</td>
<td>5.43±0.95</td>
<td>5.03±1.55</td>
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<td>2.78±0.37</td>
<td>2.43±0.07</td>
<td>1.71±0.31</td>
<td>0.48±0.07</td>
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<td>Lymph node</td>
<td>18.59±4.76</td>
<td>19.88±0.69</td>
<td>20.83±9.73</td>
<td>4.23±2.66</td>
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<td>Liver</td>
<td>19.53±1.50</td>
<td>9.47±0.51</td>
<td>4.35±1.47</td>
<td>1.74±1.73</td>
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<td>Spleen</td>
<td>10.49±4.80</td>
<td>18.65±3.56</td>
<td>9.61±3.25</td>
<td>1.41±0.24</td>
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<tr>
<td>Kidneys</td>
<td>17.24±5.25</td>
<td>29.06±1.62</td>
<td>12.44±4.44</td>
<td>3.24±0.45</td>
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<tr>
<td>Stomach</td>
<td>3.89±0.49</td>
<td>3.87±0.34</td>
<td>2.40±0.61</td>
<td>0.65±0.13</td>
</tr>
<tr>
<td>Small intestine</td>
<td>3.88±0.57</td>
<td>4.38±1.33</td>
<td>6.03±0.83</td>
<td>2.92±0.97</td>
</tr>
<tr>
<td>Colon</td>
<td>3.13±0.75</td>
<td>1.93±0.40</td>
<td>1.38±0.22</td>
<td>4.29±0.66</td>
</tr>
<tr>
<td>Femur</td>
<td>3.22±0.75</td>
<td>3.65±0.51</td>
<td>1.83±0.31</td>
<td>0.70±0.16</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>0.90±0.14</td>
<td>0.75±0.04</td>
<td>0.50±0.11</td>
<td>0.13±0.02</td>
</tr>
</tbody>
</table>

Morphometric Analysis (Intima/Media Ratio)

Samples of deendothelialized (Evans blue–positive), reendothelialized (appear lighter than normal vessel, plaque formation), and uninjured areas were biopsied (3 samples from each artery), embedded in paraffin, sectioned into 5-μm-thick slices, and stained with hematoxylin and eosin for light microscopy and histomorphometry. The sections were scanned with \(\times4\) magnification and digitized with the Image Analyst Program (Automatix). The external and internal elastic lamina and lumen/intima border were identified, and an intima/media ratio was calculated. The intima was defined as the vessel layer between the internal elastic lamina and the intima/lumen border, and the media was defined as the area between the internal and external elastic laminae. Three regions were measured for each sample.
clearance half-life is in Table 1 (% ID/g) and Table 2 (% ID/organ). The blood similar to that in the mouse, with a half-life of of 125 I-MCP-1 in whole blood ranged from 4 to 7 times at 60 minutes and 0.01% at 180 minutes. The concentration 0.05% at 1 minute after injection and decreased to 0.02% 0.05% at 180 minutes. The plasma concentration was 5.0002) (Figure 2, bottom left) and blood (0.04±0.01% ID/g, P=0.0005). The maximal ratio of plaque to blood radioactivity was 7.08. The distribution of 125 I-labeled MCP-1 in rabbit tissue 3 hours after administration is summarized in correlated with the number of macrophages by linear regression. A value of P<0.05 was considered statistically significant.

Results

Biodistribution of 125I-Labeled MCP-1 in Mice
The tissue distribution of 125I-labeled MCP-1 is summarized in Table 1 (% ID/g) and Table 2 (% ID/organ). The blood clearance half-life is ~10 minutes (% ID/organ). Blood activity decreased to 9.70% ID/g at 60 minutes and 2.86% ID/g at 180 minutes. On the basis of % ID/organ, liver, lungs, and kidneys had the highest concentration at 5 and 30 minutes after tracer administration. At 60 minutes, lymph nodes (% ID/g) had the 3 highest. At 180 minutes, the blood and small intestine had the highest concentration (% ID/organ).

Blood Clearance of 125I-MCP-1 in a Rabbit
Blood clearance of 125I-labeled MCP-1 in the rabbit is similar to that in the mouse, with a half-life of <20 minutes. The concentration of radioactivity (% ID/g) in the blood was 0.39% at 1 minute after the administration of 125I-MCP-1 and decreased to 0.10% at 60 minutes and 0.05% at 180 minutes. The plasma concentration was 0.05% at 1 minute after injection and decreased to 0.02% at 60 minutes and 0.01% at 180 minutes. The concentration of 125I-MCP-1 in whole blood ranged from 4 to 7 times greater than that in plasma (Figure 1), suggesting that 125I-MCP-1 is bound to cells.

Distribution of 125I-MCP-1 in the Injured Arteries of Rabbits
Plaque areas had significantly higher % ID/g than uninjured areas of the vessel (0.12±0.04 versus 0.02±0.006, P=0.0002) (Figure 2, bottom left) and blood (0.04±0.01% ID/g, P=0.0005). The maximal ratio of plaque to blood radioactivity was 7.08. The distribution of 125I-labeled MCP-1 in rabbit tissue 3 hours after administration is summarized in

Table 3. The kidneys had the highest concentration of radiolabel (0.94±0.13% ID/g), significantly higher than that of plaque (P<0.0001). The spleen had the second highest concentration (0.04±0.007% ID/g), which was lower than that of plaque (P=0.0005).

Intima/Media Ratio
The medial layer was similar in the intact, deendothelialized, and reendothelialized lesions. Plaque had higher intima/media ratios (2.92±1.42, n=8) than denuded (1.51±0.33, n=8) and uninjured (0.03±0.06, n=8) areas (P<0.02 and P<0.0001, respectively). The intima/media ratio in the denuded areas was significantly higher than that in the intact areas (P<0.02) (Figure 3).

Immunohistochemistry
Cells staining with RAM-11 were significantly greater in plaque (n=8) than denuded (n=8) areas (29.81±10.59 versus 17.03±10.10, P<0.05) (Figure 2, photo, center column and graph, center); few RAM-11–positive cells were observed in intact areas. RAM-11–positive cells were detected in both the media and intima of plaque regions.

Autoradiography
In the injured regions, 3 levels of radioactivity were seen (Figure 2A, and autoradiograph, B): (1) areas of regenerating endothelium, which did not stain with Evans blue dye (appear pale, marked as 3 on the photo and autoradiograph), had the most intense accumulation of 125I-MCP-1 (regions of plaque); (2) deendothelialized regions, which stain with Evans blue, corresponded to areas of moderate radioactivity (denuded areas, marked as 2 on the photo and autoradiograph); and (3) noninjured areas, which did not stain with Evans blue (marked as 1 on the photo and autoradiograph), had faint accumulation of radioactivity. Although there was some variation in the intensity of activity among different animals or different reendothelialized or deendothelialized regions, a

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</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>57.15±4.65</td>
<td>27.81±2.26</td>
<td>15.37±5.50</td>
<td>4.65±0.82</td>
</tr>
<tr>
<td>Lungs</td>
<td>7.54±0.78</td>
<td>8.45±1.14</td>
<td>3.46±1.43</td>
<td>0.33±0.05</td>
</tr>
<tr>
<td>Heart</td>
<td>0.40±0.04</td>
<td>0.26±0.05</td>
<td>0.18±0.10</td>
<td>0.04±0.01</td>
</tr>
<tr>
<td>Thyroid</td>
<td>0.10±0.02</td>
<td>0.14±0.06</td>
<td>0.09±0.02</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.12±0.06</td>
<td>0.12±0.01</td>
<td>0.07±0.02</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>Liver</td>
<td>20.80±1.48</td>
<td>11.17±0.49</td>
<td>5.69±1.75</td>
<td>1.53±0.18</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.04±0.44</td>
<td>1.92±0.25</td>
<td>0.78±0.30</td>
<td>0.15±0.03</td>
</tr>
<tr>
<td>Kidneys</td>
<td>4.66±1.44</td>
<td>9.04±0.58</td>
<td>4.04±1.50</td>
<td>1.06±0.09</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.70±0.08</td>
<td>0.59±0.03</td>
<td>0.41±0.08</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td>Small intestine</td>
<td>3.57±0.61</td>
<td>4.21±0.32</td>
<td>7.00±0.97</td>
<td>3.28±0.98</td>
</tr>
<tr>
<td>Colon</td>
<td>2.09±0.79</td>
<td>2.05±0.79</td>
<td>1.08±0.21</td>
<td>3.25±0.61</td>
</tr>
</tbody>
</table>

**Table 2. Biodistribution (% ID/organ) of 125I MCP-1 in Mice**

**Table 3. Biodistribution of 125I MCP-1 at 3 Hours After Administration**
similar pattern of $^{125}$I-MCP-1 distribution was seen in all animals. The maximum lesion:normal vessel ratio of $^{125}$I-MCP-1 was 44.8:1. The accumulation of $^{125}$I-MCP-1 in the reendothelialized areas was greater than in the deendothelialized area (6.55 ± 2.26 versus 4.34 ± 1.43 density per pixel, $P < 0.05$). The accumulation of $^{125}$I-MCP-1 correlated ($r = 0.85, P < 0.0001$) with the number of macrophages per unit area (Figure 2, graph, right).

**Discussion**

These data suggest that radiolabeled MCP-1, a cytokine for monocytes and macrophages, localizes in experimental arterial lesions induced by balloon deendothelialization and a 1% cholesterol diet in the rabbit. Sites of regenerating endothelium correlated with the highest $^{125}$I-MCP-1 and macrophage localization. MCP-1 activity in the lesions was greater than that in the blood and far greater than that in the normal arterial wall.

The ratio of whole blood to plasma suggests that the majority of circulating peptide is associated with cells. Cellular association may also explain the transient pulmonary uptake of $^{125}$I-MCP-1. The “first-pass” presentation of MCP-1 to the alveolar macrophages is at a relatively higher concentration than after hemodilution. These cells express receptors for this peptide that bind transiently because the number of receptors is not upregulated. The peptide clears slowly from the uninflamed lungs. But in atheroma, the number of receptors is markedly upregulated, causing both a high initial uptake and prolonged retention of the peptide at the lesion site.

Many radionuclide imaging approaches have been advocated to detect atherosclerotic lesions. Previous publications describe the use of radiolabeling: autologous lipoproteins $^{15-18}$; platelets and platelet-specific antibodies $^{19-21}$; agents that bind to activated glycoprotein IIb/IIIa receptors expressed on activated platelets $^{22}$; an adenine nucleotide analogue, Ap$_4$A, directed at ADP association sites on platelets $^{23}$; fibrinogen $^{24,25}$; and an IgM antibody $^{26,27}$ that recognizes proliferating arterial smooth muscle cells found in active plaque. Some of these agents, such as LDL,
platelets, and the antibody recognizing proliferating smooth muscle (Z2D3), have been tested in humans. The agents had some success identifying lesions in the carotid arteries but have not identified atheroma with a sufficient sensitivity to be clinically useful. None of these agents have demonstrated disease in coronary vessels. These studies demonstrate the remarkably dynamic nature of atheroma, however, because autologous LDL localized in human carotid lesions within hours of administration. This finding is confirmed by the localization of MCP-1 in the lesion within 180 minutes of injection.

It is not clear that 180 minutes is an optimum time to detect atheroma, however, because lesion kinetics were not evaluated in this study. A short interval between injection and sampling minimizes the opportunity for dissociation and metabolism of the peptide at the receptor site, whereas a longer interval allows more clearance of peptide from background structures, such as the lungs and blood. On the basis of these factors, the 3-hour interval was selected.

An average ratio of lesion to normal vessel of >6:1 was observed, which was substantially greater than residual activity in the blood. These data suggest that external radionuclide imaging of macrophage-rich arterial lesions may be feasible with radiolabeled MCP-1 when the agent is labeled with either $^{125}$I or $^{99m}$Tc.

In the present study, external imaging with a gamma camera was not attempted because of the low energy of $^{125}$I and the extremely low dose of $^{125}$I-MCP-1. Identifying vulnerable plaque is clinically important because these lesions are more likely to erode or rupture the fibrous cap, resulting in acute coronary occlusion. Although myocardial perfusion imaging is widely used to detect ischemic lesions, the location of ischemia on a stress perfusion scan has a limited correlation with the location of a subsequent infarction. Similarly, serial coronary arteriograms demonstrate populations of lesions that undergo gradual but continuous progression over time and lesions that may remain stable for long intervals. Progression of atheroma often occurs in lesions with a high concentration of monocytes and macrophages.

These observations suggest that quantitative evaluation of macrophages in lesions may be useful to identify those lesions with a high risk of plaque rupture. The present study suggests radiolabeled MCP-1 as a candidate radiotracer to assess plaque vulnerability on the basis of the expression of MCP-1 receptors on macrophages that have localized at the lesion site. Large lesions located in superficial vessels may be detectable by external radionuclide imaging. The small size of many coronary plaques, the depth of the heart, and the fact that it is in motion, however, make external imaging of these lesions challenging with today’s technology. To detect small atheroma, it may be necessary to place the detector close to the plaque, which will require an intravascular radionuclide detection device.

Conclusions

$^{125}$I-MCP-1 localizes in macrophage-rich experimental atherosclerotic lesions in vivo.

Acknowledgment

Dr Ohtsuki was supported in part by a generous grant from the Uehara Memorial Foundation.

References


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Circulation. 2001;104:203-208
doi: 10.1161/01.CIR.104.2.203

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