Elevated Circulating Levels of Monocyte Chemoattractant Protein-1 in Patients With Restenosis After Coronary Angioplasty

Francesco Cipollone, Matteo Marini, Maria Fazia, Barbara Pini, Annalisa Iezzi; Marcella Reale, Leonardo Paloscia, Guido Materazzo, Erminio D’Annunzio, Pio Conti, Francesco Chiarelli, Franco Cuccurullo, Andrea Mezzetti

Abstract — Inflammation plays a pathogenic role in the development of restenosis after percutaneous transluminal coronary angioplasty (PTCA). Monocyte chemoattractant protein-1 (MCP-1) is a potent chemoattractant of monocytes; however, its role in the pathophysiology of restenosis is still unclear. We set out to investigate the role of MCP-1 in restenosis after PTCA. In addition, we tested the hypothesis that MCP-1 exerts its effect, at least in part, by inducing $O_2^-$ generation in circulating monocytes. Plasma levels of MCP-1 were measured before and 1, 5, 15, and 180 days after PTCA in 50 patients (30 males and 20 females, aged 62 ± 5 years) who underwent PTCA and who had repeated angiograms at 6-month follow-up. Restenosis occurred in 14 (28%) patients. The MCP-1 level was no different at baseline between patients with or without restenosis. However, after the procedure, restenotic patients, compared with nonrestenotic patients, had statistically significant ($P < 0.0001$) elevated levels of MCP-1. In contrast, plasma levels of other chemokines, such as RANTES and interleukin-8, did not differ between the 2 groups after PTCA. Higher MCP-1 throughout the study was correlated with restenosis. Moreover, increased MCP-1 was significantly correlated with increased monocyte activity, as reflected by enhanced $O_2^-$ generation. Finally, multivariate regression analysis showed that the MCP-1 plasma level measured 15 days after PTCA was the only statistically significant independent predictor of restenosis ($b = 0.688$, $P < 0.0001$). This study suggests that MCP-1 production and macrophage accumulation in the balloon-injured vessel may play a pivotal role in restenosis after PTCA. MCP-1 may induce luminal renarrowing, at least in part, by inducing $O_2^-$ release in monocytes. Further understanding of the mechanism(s) by which MCP-1 is produced and acts after arterial injury may provide insight into therapies to limit the progression of atherosclerosis and restenosis after balloon angioplasty. (Arterioscler Thromb Vasc Biol. 2001;21:327-334.)

Key Words: angioplasty ■ restenosis ■ monocyte chemoattractant protein-1 ■ superoxide anion ■ monocytes

Restenosis after percutaneous transluminal coronary angioplasty (PTCA) is still a major clinical problem. It occurs in 30% to 50% of patients who undergo PTCA and limits its long-term success.1 The pathophysiology of restenosis has not yet been elucidated. It is supposed that vessel injury may initiate an inflammatory response that accelerates the recruitment of mononuclear monocytes, which play a crucial role in healing and remodeling.2 Oxidative stress mediated by increased levels of superoxide anion ($O_2^-$) also appears to be an important component of the vessel response to injury,3 possibly contributing to restenosis after coronary interventions. Thus, an evolving concept is that activated blood monocytes may contribute to intimal proliferation by the production of reactive oxygen species (ROS), cytokines, and growth factors.4,5

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Monocyte chemoattractant protein-1 (MCP-1) is the prototype of the C-C chemokine $\beta$ subfamily and exhibits its most potent chemotactic activity toward monocytes6 and T lymphocytes.7 In addition to promoting the transmigration of circulating monocytes into tissues, MCP-1 exerts various effects on monocytes, including superoxide anion induction,8 cytokine production, and adhesion molecule expression.9 MCP-1 expression is induced by inflammatory cytokines, peptide growth factors, or minimally modified LDL in monocytes, endothelial cells, and vascular smooth muscle cells.10,11 Elevated levels of MCP-1 have been found in patients with myocardial infarction12 and heart failure,8 as well as after myocardial reperfusion.13 Moreover, increased MCP-1 has been detected in the aorta after balloon injury11

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From the Department of Medicine and Aging (F.C., M.M., M.F., B.P., A.I., F. Chiarelli, F. Cuccurullo, A.M.) and the Department of Biomedical Science (M.R., P.C.), University of Chieti “G D’Annunzio” School of Medicine, Chieti, Italy, and the Division of Cardiology (L.P., G.M., E.D.), “Spirito Santo” Hospital, Pescara, Italy.
Correspondence to Andrea Mezzetti, MD, Centro per la Prevenzione dell’Aterosclerosi, la Diagnosi e Terapia dell’Ipertensione Arteriosa e delle Dislipidemie, Nuovo Policlinico SS. Annunziata, Via dei Vestini 66, 66013 Chieti, Italy. E-mail mezzetti@unich.it
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Initially successful angioplasty was followed by persistent abrupt closure; if a Q-wave infarction occurred in the territory of the dilated artery; if angioplasty was unsuccessful, necessitating emergency revascularization; if the results of angioplasty were suboptimal and a stent was implanted; or if follow-up angiography was lost. None of the participating subjects were taking vitamins, dietary supplements, or drugs with known antioxidant activity. All patients received aspirin (100 mg daily) for the entire study period. Informed consent was obtained from each subject. The study protocol was approved by the Institutional Ethical Committee.

**Angioplasty Procedure and Follow-Up Evaluation**

Balloon angioplasty was performed according to standard techniques. Patients were discharged with aspirin and any other necessary medication. They were specifically asked not to take additional vitamins and mineral supplements, and the American Heart Association Step 1 Diet was taught to all patients. After successful angioplasty, patients returned for a clinical control after 15 days and 1, 3, and 6 months. Patients were readmitted for follow-up coronary angiography 6 months after angioplasty.

**Quantitative Coronary Angiography**

The 4 coronary arteriograms (obtained before the procedure, immediately after the procedure, 15 minutes after the procedure, and at the 6-month follow-up visit) were analyzed by an experienced cardiologist using the Philips H-4000 Quantitative Coronary Analysis. Measurements were made in a single projection, showing the most severe stenosis. Whenever possible, the same projection was used in all 4 arteriograms to allow more accurate comparison. The variation among repeated measurements of the percentage of stenosis was 7% when frames recorded 1 to 6 months apart were analyzed.

**Definitions**

Restenosis in a previously successfully dilated lesion was defined as recurrent lumen diameter stenosis >50% at follow-up angiography, as determined by quantitative coronary angiography. The continuous variable luminal loss was defined as the change in minimal lumen diameter (MLD) during follow-up normalized for vessel size, according to the following equation: relative loss (RLOSS) = [(postintervention MLD - follow-up MLD)/vessel size] x 100%; the equation reflects the degree of luminal narrowing. The vessel size is the value of the reference diameter function at the minimal position of the obstruction.

**Blood Collection**

For serum sampling, blood was collected from each patient before and 24 hours after PTCA and drawn into pyrogen-free collection tubes without additives. Tubes were immediately immersed in melting ice and allowed to clot for 1 hour before centrifugation at 1000g for 10 minutes. For plasma sampling, blood was collected from each patient before PTCA and 1, 5, 15, and 180 days afterward. Blood was drawn into pyrogen-free test tubes containing EDTA as anticoagulant. Tubes were immediately immersed in melting ice and centrifuged (1000g at 4°C for 15 minutes) within 15 minutes after sampling. Multiple aliquots of serum and plasma were stored at −80°C until analysis. Samples were frozen and thawed only once.

**Isolation of Blood Monocytes**

The isolation of peripheral monocytes from 5 healthy blood donors was assessed by an established method. Briefly, monocytes were isolated from the buffy-coat fraction, which was reconstituted with PBS to 20 mL, layered on top of a Lymphoprep gradient (Nycomed), and centrifuged at 800g for 10 minutes at room temperature. The fraction containing the mononuclear leukocytes was washed with PBS. Next, the erythrocytes were lysed in ammonium chloride solution, and the remaining mononuclear leukocytes were washed once with ammonium chloride solution and once with PBS and resuspended in RPMI 1640 (GIBCO) with 2% FCS. The percentage of monocytes in this fraction was determined from cytopsin preparations and, in general, amounted to 22%.
Superoxide Anion (O$_2^-$) Assay
The purified mononuclear cells (3×10$^6$/mL, 200 μL per well) were cultured in 96-well trays (Costar) in RPMI 1640 with l-glutamine (GIBCO) for 20 hours with 20% of serum from patients or healthy control subjects. For processing of serum, 40 μL of serum from each patient or control subject was added to monocyte culture immediately after thawing, at the start of the culture period. In some experiments, neutralizing monoclonal antibodies against MCP-1 (goat anti-human MCP-1, final concentration 50 μg/mL, R&D System) or control mouse IgG (final concentration 50 μg/mL, R&D System) were also added to cell culture. After 20 hours in culture, the generation of O$_2^-$ from adherent monocytes was measured by the superoxide dismutase-inhibitable reduction of cytochrome c. The O$_2^-$ generation was expressed as nanomoles per 60 minutes per 10$^6$ monocytes.

Enzyme Immunoassays
Concentrations of MCP-1, RANTES, and interleukin-8 (IL-8) in plasma were determined in duplicate by specific enzyme immunoassays (BioSource) according to the manufacturers' descriptions. At our laboratory, the intra-assay and interassay coefficients of variation were <8%.

Statistical Analysis
For clinical data, variables were compared by use of the χ$^2$ test. An ANOVA for repeated measures followed by multiple comparison test (Scheffé test) was performed to test the changes in biochemical variables measured over time. Differences in biochemical variables between the restenotic and nonrestenotic groups at each collection time were analyzed by the Student unpaired t test. The correlation between MCP-1 and O$_2^-$ and the strength of the association of late lumen loss with MCP-1 and monocyte O$_2^-$ generation was assessed by linear regression analysis. Each variable that proved to be statistically significant in the univariate regression analysis was assessed by multiple regression to establish whether it was an independent risk factor for late lumen loss. Multiple regression analysis was adjusted for potential confounders (class of angina, cigarette smoking, hypercholesterolemia, diabetes mellitus, hypertension, age, diet, concomitant therapy, or procedural methods). The data are expressed as proportions or as mean±SD. Statistical significance was considered to be indicated by a value of P<0.05. All calculations were performed by using the computer program SPSS 8.0.

Results
Clinical Course
No patient developed sudden death during the study, and there were no bleeding complications that required blood transfusion. Restenosis occurred in 14 (28%) of 50 patients.

Circulating Levels of MCP-1 in Patients With or Without Restenosis After PTCA
The time course of MCP-1 biosynthesis during the study is depicted in Figure 1. In both groups of patients, MCP-1 was significantly increased when measured after PTCA throughout the study (P<0.0001, ANOVA). There was no notable difference between patients with or without restenosis before PTCA (480±42 versus 470±69 [mean±SD] pg/mL, respectively). Nevertheless, after PTCA in restenotic patients, enhanced MCP-1 persisted as statistically significant (P<0.0001) throughout the study, whereas in the nonrestenotic patients, MCP-1 levels normalized 15 days after the procedure (Figures 1 and 2). Thus, MCP-1 was significantly higher (P<0.0001) in the patients with restenosis at 1 (816±176 versus 618±102 pg/mL), 5 (755±158 versus 594±111 pg/mL), 15 (715±80 versus 450±64 pg/mL, Figure 2), and 180 (712±94 versus 453±76 pg/mL) days after PTCA.

Baseline levels of plasma MCP-1 averaged 498±54 (mean±SD) pg/mL in the patients with unstable angina (n=35). This rate of biosynthesis was significantly higher (P<0.0001) than that in the patients with stable angina (412±32 [mean±SD] pg/mL, n=15). However, differences in MCP-1 biosynthesis between patients with or without restenosis could not be accounted for by class of angina (unstable versus stable), cigarette smoking, hypercholesterolemia, diabetes mellitus, hypertension, age, diet, concomitant therapy, or procedural methods, because these potential confounders were equally represented in the 2 groups (Table 1).
Moreover, to explore the specificity of the observed response in the broader group of chemokines and to determine whether chemokines targeting other cell types could be also involved in the pathophysiology of restenosis, we analyzed the time course of RANTES and IL-8 after PTCA. In both groups of patients, RANTES and IL-8 were significantly increased when measured 1 and 5 days after PTCA, but they achieved normalization 15 days after the procedure, with no notable differences between patients with or without restenosis (Figure 3).

**Relationship Between MCP-1 and Generation of ROS From Monocytes**
MCP-1 has been reported to induce enhanced ROS generation in monocytes. To further examine the relation between MCP-1 and monocyte activity in patients who had undergone PTCA, the effect of serum collected from patients before and 24 hours after PTCA on the generation of \( \cdot \text{O}_2^- \) in monocytes was studied. In this experiment, 50 nonhospitalized healthy volunteers (28 males and 22 females, aged 60±8 years) without any clinically detectable pathological condition were also studied as a control group. Monocytes from 5 healthy blood donors were evaluated for spontaneous and PMA-stimulated \( \cdot \text{O}_2^- \) generation after they were cultured for 20 hours in medium supplemented with either 20% serum from 50 patients subjected to PTCA with broad-spectrum MCP-1 levels (range 270 to 1190 pg/mL) or 20% serum from 50 healthy blood donors (<300 pg/mL). Monocytes spontaneously generated considerable levels of \( \cdot \text{O}_2^- \) when they were cultured with serum collected from patients before and 24 hours after PTCA; the levels were significantly higher in the subset of patients with restenosis (8.6 ± 2 versus 5.9 ± 2 nmol/60 minutes per 10^6 monocytes \([P=0.002]\) and 10.2 ± 2 versus 8 ± 2 nmol/60 minutes per 10^6 monocytes \([P=0.001]\), respectively; Figure 4). In contrast, no detectable spontaneous \( \cdot \text{O}_2^- \) generation was measured in monocytes cultured with serum from healthy donors (Figure 4). The stronger stimulatory effect of serum from

**Figure 3.** Time course of plasma IL-8 (top graph) and RANTES (bottom graph) in patients with or without restenosis before (control) and 1, 5, 15, and 180 days after PTCA. Bars and vertical lines represent mean±SD values.
Restenotic patients on the spontaneous generation of $O_2^-$ in monocytes was blocked by an inhibitory monoclonal antibody for MCP-1 ($8 \pm 2$ versus $6 \pm 1.2$ nmol/60 minutes per $10^6$ monocytes [$P<0.05$] and $10.2 \pm 2$ versus $8.1 \pm 1$ nmol/60 minutes per $10^6$ monocytes [$P<0.03$], respectively, for samples collected before and 24 hours after PTCA), thus confirming the critical role of MCP-1 in enhanced monocyte $O_2^-$ generation (Figure 4).

PMA-stimulated $O_2^-$ generation was also enhanced when monocytes were cultured with serum from patients; again, the most marked effect of serum was at the highest MCP-1 level, which was statistically significant ($P=0.003$) in the samples collected 24 hours after PTCA ($57 \pm 5$ versus $45 \pm 6$ nmol/60 minutes per $10^6$ monocytes, Figure 4). Accordingly, a strongly positive correlation ($P<0.0001$) between plasma MCP-1 and either spontaneous or stimulated monocyte $O_2^-$ generation was found before PTCA ($R^2=0.691$ and $R^2=0.404$, respectively) and after PTCA ($R^2=0.284$ and $R^2=0.420$, respectively; Figure 5).

**Variables Predictive for Late Lumen Loss**

To investigate whether MCP-1 and MCP-1–dependent oxidant stress could contribute to luminal renarrowing after PTCA, we assessed the association between MCP-1 and monocyte $O_2^-$ generation with the degree of luminal renarrowing (RLOSS) at 6 months after PTCA. RLOSS showed a positive correlation ($P<0.0001$) with plasma MCP-1 levels measured 1 ($R^2=0.335$), 5 ($R^2=0.357$), 15 ($R^2=0.722$), and 180 ($R^2=0.601$) days after PTCA. Moreover, a positive correlation with either spontaneous and stimulated monocyte $O_2^-$ generation was found 24 hours after PTCA ($R^2=0.171$ [P=0.003] and $R^2=0.239$ [P=0.0001], respectively). Finally, multivariate regression analysis showed that only the plasma level of MCP-1 measured 15 days after PTCA was a statistically significant independent predictor for luminal renarrowing after PTCA ($\beta=0.688$, $P<0.0001$; Figure 6).
To our knowledge, the present study is the first to provide evidence in humans of increased MCP-1 levels early and as long as 6 months after PTCA in patients developing restenosis and the first to identify MCP-1 as a powerful independent predictor of late lumen loss after coronary angioplasty.

In the present study, patients who underwent PTCA experienced a critical increase in circulating MCP-1 after the procedure, which was significantly more evident and prolonged in those with restenosis (Figure 1). In fact, whereas in the patients without restenosis, MCP-1 plasma levels returned to baseline after 15 days (Figures 1 and 2), in the patients with restenosis, the changes persisted throughout the study. Thus, the differences between the 2 groups became evident 24 hours after PTCA and persisted as statistically significant during the whole period of examination (Figure 1).

The striking effect of angioplasty in increasing MCP-1 raises the question of the origin of this chemokine. MCP-1 is produced by monocytes, endothelial cells, and fibroblasts. Macrophages and smooth muscle cells, the major types of cells left in the atherosclerotic lesion after PTCA, can also elaborate MCP-1. Several studies have shown that PTCA results in persistent platelet activation. Activated platelets have been found to stimulate MCP-1 production in monocytes through enhanced RANTES secretion and direct platelet-monocyte contact mediated by P-selectin expression on the platelet surface. Such a mechanism for enhanced MCP-1 expression in leukocytes has recently been found to be operative in patients with acute myocardial infarction, and it is conceivable that such a platelet-monocyte interaction may also contribute to the enhanced MCP-1 levels in patients who undergo coronary angioplasty. Moreover, the observation of Aukrust et al, who showed that monocytes isolated from patients with heart disease released higher amounts of MCP-1 than did cells from healthy control subjects and may therefore contribute to the elevated MCP-1 levels in the setting of pathological conditions, is of particular interest.

Whatever the cellular source(s), the enhanced levels of MCP-1 may indirectly and directly have important pathophysiological consequences in patients who undergo PTCA, as shown by the statistically significant relationship between MCP-1 and late lumen loss at the 6-month follow-up (Figure 6).

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Whatever the cellular source(s), the enhanced levels of MCP-1 may indirectly and directly have important pathophysiological consequences in patients who undergo PTCA, as shown by the statistically significant relationship between MCP-1 and late lumen loss at the 6-month follow-up (Figure 6). MCP-1 has chemotactic activity for monocytes and lymphocytes and has been postulated to be a major signal for the accumulation of mononuclear leukocytes after vessel injury. There are several reports suggesting that infiltration of lymphocytes and monocytes into the vessel wall after PTCA by various mechanisms may lead to progressive luminal narrowing. By playing a pivotal role in the
recruitment of these cells into sites of inflammation. MCP-1 may thus indirectly play an important role in the pathogenesis of restenosis after PTCA. The possible importance of MCP-1 for the induction of intimal hyperplasia was recently illustrated by Stark et al., who showed that upregulation of MCP-1 gene expression in vein grafts resulted in the recruitment of monocytes and tissue macrophages to the vein wall, which led to obliterator stenosis and graft failure. Moreover, Furukawa et al. showed that neutralization of MCP-1 before and immediately after arterial injury may be effective in preventing intimal hyperplasia.

MCP-1 may also more directly contribute to restenosis. In the present study, we found that the raised MCP-1 levels in serum from patients who developed restenosis after PTCA had enhancing effects on spontaneous ROS generation in monocytes (Figure 4). Thus, if this enhancement also occurs in vivo, it may be directly involved in the response to vascular injury. In fact, previous studies suggest that after vascular injury, an oxidizing environment must exist in the plasma and in the vessel wall that may influence vascular remodeling. The generation of ROS has profound and wide-ranging effects that can dramatically increase vascular toxicity and initiate a cascade of molecular and cellular responses. Superoxide anions can react with NO, reducing the vasoreactivity of NO and diminishing the response to endothelium-dependent vasodilators via formation of peroxynitrite anion (ONOO\(^{-}\)), a highly reactive intermediate with strong cytotoxic potency. Furthermore, the damaging free radicals in patients with enhanced oxidant stress may cause either direct arterial wall injury or may initiate and/or accelerate secondary processes, including inflammatory gene induction, activation of phagocyte–platelet–endothelial cell interactions, protein peroxidation, and depletion of antioxidants such as vitamins C and E. In the present study, we have also demonstrated the specific role of MCP-1 in enhanced O\(_2\)\(^{-}\) generation in patients who developed restenosis after PTCA by using neutralizing antibody against human MCP-1 (Figure 4). In fact, MCP-1 neutralization reduced the generation of O\(_2\)\(^{-}\) to the level observed in patients without restenosis. However, O\(_2\)\(^{-}\) generation was still higher with respect to healthy subjects, thus suggesting that other stimuli are also involved in the complex scenario of acute vascular response after PTCA.

Enhanced O\(_2\)\(^{-}\) generation may also amplify MCP-1 production. In fact, many vascular biologists now accord a central role to augmented transcription of several atherosclerosis-related genes by the oxidant-sensitive regulatory pathway involving nuclear factor \(\kappa B\). Exposure to extracellular O\(_2\)\(^{-}\) removes the inhibitory effect of NO on MCP-1 expression, activates the nuclear factor-\(\kappa B\) regulatory complex, and triggers the transcription of genes that encode certain growth factors, adhesion molecules, chemoattractant cytokines (such as MCP-1), and enzymes that can influence extracellular matrix metabolism. Thus, increased O\(_2\)\(^{-}\) generation in monocytes may further enhance the synthesis of MCP-1 in these cells through an autocrine mechanism, possibly representing a vicious circle implicated in restenosis after balloon angioplasty. Moreover, O\(_2\)\(^{-}\) generation might evoke a secondary cytokine and growth factor response from other types of cells in the lesion, including smooth muscle cells, fibroblasts, and endothelial cells, and establish a positive, self-stimulatory, paracrine feedback loop, amplifying and sustaining the proliferative response.

We did not perform any measurements in patients who underwent stenting after PTCA; therefore, the generalizability of these results to the overall population of patients who are treated with percutaneous coronary intervention is uncertain and requires additional studies. However, when the present study was started, the efficacy of stenting was supported by a randomized trial only in highly selected patients with predominantly simple coronary stenosis, whereas there was not yet concrete evidence regarding the outcome among unselected patients. Another limitation is that the predictive value of MCP-1 measured 15 days after PTCA found in the present study needs to be considered cautiously because of the relatively small number of patients (and events). However, the prognostic value of this marker, if confirmed in larger clinical studies, could contribute to optimizing therapeutic resources in the complex scenario of interventional cardiology.

In conclusion, the present study supports the hypothesis that upregulation of MCP-1 gene expression after coronary angioplasty results in the recruitment of monocytes and tissue macrophages to the arterial wall, possibly contributing to a number of restenoses after PTCA. Moreover, these results suggest that the causative role of MCP-1 in restenosis is mediated, at least in part, by increased generation of O\(_2\)\(^{-}\). Therefore, further understanding of the mechanism(s) by which MCP-1 is produced and acts after arterial injury may provide insight into therapies to limit the progression of atherosclerosis and restenosis after balloon angioplasty.

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References


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