Circulating Monocyte Chemoattractant Protein-1 and Early Development of Nephropathy in Type 1 Diabetes

FRANCESCO CHIARELLI, MD[1]
FRANCESCO CIPOLLONE, MD[2]
ANGELIKA MOHN, MD[1]
MATTEO MARINI, PHD[2]
ANNALISA IEZZI, PHD[1]
MARIA FAZIA, PHD[2]
STEFANO TUMINI, MD[1]
DOMENICO DE CESARE, TCH[2]
MARIAPINA POMILIO, MD[1]
SANTE D. PIERDOMENICO, MD[2]
MARIO DI GIOACCHINO, MD[2]
FRANCO CUCCURULLO, MD[2]
ANDREA MEZZETTI, MD[2]

OBJECTIVES — To investigate the possible role of hyperglycemia-dependent monocyte chemoattractant protein (MCP)-1 biosynthesis in the pathophysiology of early nephropathy in type 1 diabetes.

RESEARCH DESIGN AND METHODS — We studied 30 patients with type 1 diabetes (15 with and 15 without microalbuminuria) compared with matched healthy control subjects. Plasma MCP-1 and plasma oxidant status (vitamin E, fluorescent products of lipid peroxidation [FPLPs], malondialdehyde [MDA]), HbA_{1c}, and albumin excretion rate [AER]) were evaluated at baseline. Furthermore, MCP-1, vitamin E, AER, and HbA_{1c} were also analyzed in the microalbuminuric diabetic patients and in the healthy volunteers after 8 weeks of high-dose (600 mg b.i.d.) vitamin E treatment.

RESULTS — FPLPs, MDA, and MCP-1 were significantly higher, whereas vitamin E was significantly lower in patients with microalbuminuria and poorer glycemic control as compared with normoalbuminuric patients and healthy control subjects. Plasma MCP-1 was positively correlated with HbA_{1c}, FPLPs, MDA, and AER, whereas plasma MCP-1 showed an inverse correlation with vitamin E. Interestingly, both MCP-1 and AER decreased significantly after vitamin E treatment, despite no changes in HbA_{1c} values.

CONCLUSIONS — This study suggests that prolonged hyperglycemia may lead to early renal complications in type 1 diabetes by inducing MCP-1 biosynthesis via enhanced oxidative stress. Long-term treatment of high-dose vitamin E significantly decreased MCP-1, thus providing a rationale basis for evaluating vitamin E supplementation as therapy adjuvant to conventional insulin treatment in type 1 diabetic patients in whom an acceptable glycemic control is difficult to achieve despite appropriate insulin treatment.

Diabetes Care 25:1829–1834, 2002

Persistent microalbuminuria (albumin excretion rate [AER] >20 μg/min) is regarded as the earliest clinical sign of incipient diabetic nephropathy (1). A causal relationship between chronic hyperglycemia and diabetic microvascular disease (2) has now been definitively established by data from a prospective controlled clinical study (3). However, the pathophysiological pathway(s) by which hyperglycemia may contribute to the development of nephropathy in diabetes is not clearly understood. Among the sequelae of hyperglycemia, excess oxidative stress has captured considerable attention as a potential mechanism of kidney disease (4).

In diabetic nephropathy, an increase in both intraglomerular pressure and extracellular matrix protein results in basal membrane thickening, mesangial proliferation, and glomerular hypertrophy (5). These changes reduce glomerular filtration surface and function and can progress to glomerulosclerosis. At this regard, fibroblast activation and matrix production stimulated by inflammatory cytokines may represent an important mechanism contributing to diabetic nephropathy. In fact, glomerular infiltration of inflammatory cells is a common finding in diabetic nephropathy and is mostly dependent on recruitment of cells from the bloodstream (6). Thus, monocyte recruitment is associated with increased extracellular matrix deposition and may be stimulated by several chemotactic factors (7).

Monocyte chemoattractant protein (MCP)-1 is a C-C chemokine that exhibits its most potent chemotactic activity toward monocytes (8) and T-cells (9). In addition to promoting the transmigration of circulating monocytes into tissues, MCP-1 exerts various effects on monocytes, including the induction of superoxide anion (10), cytokines production, and adhesion molecule expression (11). MCP-1 biosynthesis is induced by inflammatory cytokines, or oxidatively modified LDL (LDLox) in monocytes, endothelial cells, and vascular smooth muscle cells (12). Furthermore, it has recently been shown that LDLox found in diabetic patients have a potent biological ability to induce MCP-1 in endothelial cells (13,14). Interestingly, it has been recently suggested that hyperglycemia can induce MCP-1 gene expression in nucleated cells

From the [1]Clinica Pediatrica, University of Chieti, Chieti, Italy; and the [2]Patologia Medica, University of Chieti, Chieti, Italy.

Address correspondence and reprint requests to Francesco Chiarelli, Clinica Pediatrica, Ospedale Policlinico, Via dei Vestini 5, I-66100 Chieti, Italy. E-mail: chiarelli@unich.it.

Received for publication 23 November 2001 and accepted in revised form 2 July 2002.

F.Ch. and F.Ci. contributed equally to this work.

Abbreviations: AER, albumin excretion rate; FPLP, fluorescent product of lipid peroxidation; HOPE, Heart Outcomes Prevention Evaluation; LDLox, oxidatively modified LDL; MCP, monocyte chemoattractant protein; MDA, malondialdehyde; ROS, reactive oxigen species.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—*Clinical characteristics of patients*

Variables	Group 1	Group 2	Group 3
Patients (*n*)	15	15	15
Age (years)	18.6 ± 4.1	18.5 ± 3.9	18.4 ± 4.0
Sex (F/M)	7/8	8/7	7/8
BMI (kg/m^2)	24.2 ± 3.9	24.7 ± 4.7	23.8 ± 3.9
Diabetes duration (years)	12.5 ± 2.5	11.2 ± 3.0	—
HbA_{1c} (%)	9.6 ± 1.4*	7.3 ± 0.9†	5.4 ± 0.6
Insulin requirement (units · kg^{-1} · day^{-1})	1.2 ± 0.3	1.0 ± 0.4	—
SBP (mmHg)	116 ± 13.5	114 ± 13.7	111 ± 12.2
DBP (mmHg)	68 ± 6.4	68 ± 8.6	65 ± 7.9
AER (μg/min)	84.2 ± 34.4*	10.4 ± 0.4	4.1 ± 0.3
Cholesterol (mmol/l)	4.2 ± 1.2	4.0 ± 1.3	3.9 ± 1.1

Data are means ± SD. DBP, diastolic blood pressure; SBP, systolic blood pressure. *$P < 0.05$ vs. both groups; $P < 0.05$ vs. group 3.

(15,16). Thus, the weight of the available evidence indicates that MCP-1 is a key factor initiating the inflammatory process of diabetic nephropathy and sustaining the extracellular matrix deposition and mesangial cell proliferation.

Thus, in the present study, we set out to investigate the possible role of MCP-1 in the development of early nephropathy in patients with type 1 diabetes. In addition, we studied the relationship between poor glycemic control and MCP-1 generation and evaluated the role of plasma antioxidant vitamin E on MCP-1 expression.

RESEARCH DESIGN AND METHODS

Patients

The study was carried out in 30 of 48 consecutive young type 1 diabetic patients (aged 19 ± 4 years) subdivided into two groups according to the presence of persistent microalbuminuria: 15 patients with microalbuminuria (group 1) and 15 patients without microalbuminuria (group 2). Persistent microalbuminuria was defined as an AER between 20 and 200 μg/min in two of three overnight urine collections performed over 6 months. Moreover, 15 healthy volunteers were studied as a control group (group 3). The three groups were carefully matched to minimize potential confounders (Table 1). The study protocol was approved by the Institutional Ethical Committee.

Design of the study

A cross-sectional comparison of circulating MCP-1 and oxidant status was performed between the three groups of study. In addition, to assess the potential influence of plasma vitamin E on MCP-1 biosynthesis, one additional intervention study was performed on the 15 microalbuminuric patients and the 15 healthy volunteers. Upon admission, these subjects were treated with vitamin E (DL-α-tocopherol acetate, Ephynal; Roche) 600 mg b.i.d. for 8 weeks.

MCP-1 assay

Concentrations of plasma MCP-1 were determined in triplicate by enzyme-linked immounosorbent assay (ELISA) (Biosource International, Camarillo, CA) as previously described (10). The influence of vitamin E, insulin, and glucose on assay determinations was studied by measuring and comparing plasma samples spiked with different doses of vitamin E, insulin, or glucose. We found no cross-reactivity for vitamin E up to 1 μg/ml, insulin up to 1 μg/ml, and glucose up to 1 μg/ml.

Generation of MCP-1 in monocytes in vitro

Peripheral blood monocytes from five healthy blood donors were isolated and cultured as previously reported (10). The purified mononuclear cells (3×10^5/ml; 200 μl/well) were incubated for 20 h with 20% of serum obtained from microalbuminuric diabetic patients before or after vitamin E supplementation or from healthy control subjects. In some experiments, vitamin E (25 μmol/l), glucose (5 or 25 mmol/l), and mannitol (25 mmol/l) were also added to cell culture. After 20 h in culture, the generation of MCP-1 from adherent monocytes was measured as described above.

Assessment of oxidant status

Lipid peroxidation in native LDL, plasma lipid peroxide content, and plasma vitamin E were evaluated as previously reported (17,18).

Statistical analysis

For the clinical data, variables were compared with the use of a χ^2 test. An ANOVA was performed with the Kruskal-Wallis method. Subsequent pairwise comparisons were made with the Mann-Whitney *U* test with corrections for multiple comparisons. Changes after treatment were analyzed with the Wilcoxon test. Simple linear regression was used for testing the association between variables of interest. Statistical analysis was performed using the SPSS 10.0.5 software.

RESULTS

Glycemic control

Mean HbA_{1c} values were significantly higher in microalbuminuric diabetic patients than in normoalbuminuric diabetic patients (9.6 ± 1.4 vs. 7.3 ± 0.93%, $P < 0.05$) (Table 1).

Circulating MCP-1 levels

Plasma MCP-1 (pg/ml, mean ± SD) was significantly higher ($P = 0.004$) in the microalbuminuric patients (91 ± 10) with respect to the normoalbuminuric patients (69 ± 6) and healthy volunteers (70 ± 8) (Fig. 1*A*). In contrast, we found no significant differences between the normoalbuminuric and the control groups. These results are unlikely to be influenced by number of circulating monocytes, because we did not find any significant difference among the three groups in the blood monocyte count at baseline.

Circulating oxidant status

Baseline plasma vitamin E (μmol/l, mean ± SD) was significantly lower ($P < 0.0001$) in microalbuminuric patients (20 ± 3) than in normoalbuminuric subjects (34 ± 5) and healthy volunteers (35 ± 4) (Fig. 1*B*). In contrast, we detected no significant difference between the normoalbuminuric and control groups.

Furthermore, oxidative burden was enhanced in microalbuminuric with re-

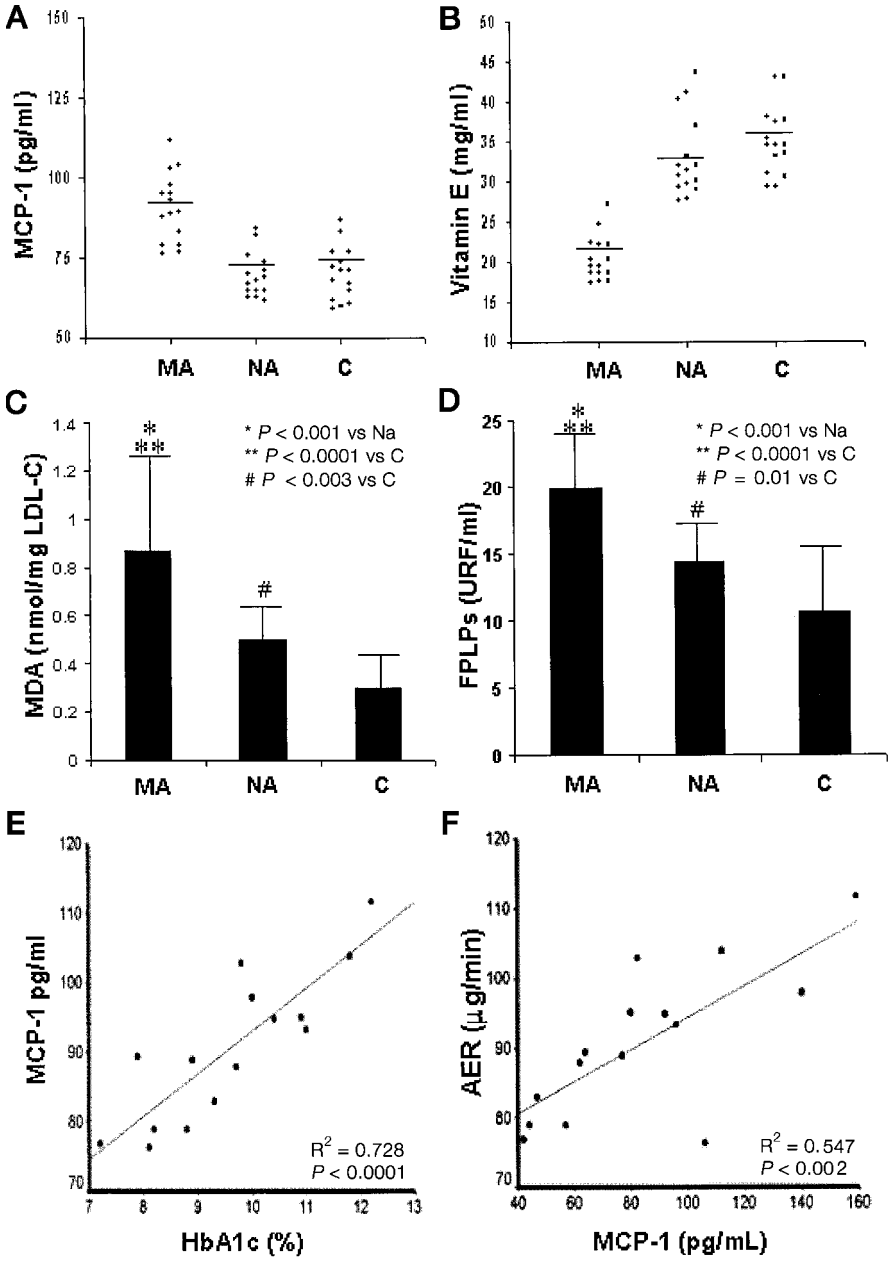

Figure 1—A: *MCP-1 concentrations in diabetic patients with microalbuminuria (MA) and without (NA) microalbuminuria and in healthy control subjects (C).* B: *Vitamin E plasma levels in diabetic patients with (MA) and without (NA) microalbuminuria, and in healthy control subjects. Each dot in* A *and* B *is representative of single measurement. Solid bars indicate mean value.* C: *MDA levels in patients with and without microalbuminuria and in healthy control subjects.* D: *FPLPs levels in patients with and without microalbuminuria and in healthy control subjects. Values in* C *and* D *are expressed as mean ± SD.* E: *Relation between MCP-1 concentrations and HbA_{1c} in patients with diabetes and microalbuminuria.* F: *Relation between MCP-1 concentrations and AER in patients with diabetes and microalbuminuria.*

spect to normoalbuminuric patients, as reflected by significantly higher levels of malondialdehyde (MDA) (0.87 ± 0.4 vs. 0.50 ± 0.08 nmol · MDA^{-1} · mg^{-1} LDL cholesterol, $P < 0.001$, Fig. 1*C*) and fluorescent products of lipid peroxidation (FPLPs) (20 ± 3 vs. 14 ± 3 URF/ml, $P < 0.0001$, Fig. 1*D*). Finally, a further difference in MDA and FPLPs levels was detected between normoalbuminuric patients and healthy control subjects (0.50 ± 0.08 vs. 0.3 ± 0.1, $P < 0.003$; and 14 ± 3 vs. 10 ± 2, $P = 0.01$, respectively).

Associations

A positive association was found between HbA_{1c} and AER ($R^2 = 0.478$, $P = 0.004$) in microalbuminuric patients, thus confiming that persistent hyperglycemia may influence the evolution of diabetic nephropathy. Next, in agreement with the hypothesis that poor glycemic control may lead to nephropathy by inducing an oxidant-dependent MCP-1 generation, we observed both in microalbuminuric and in normoalbuminuric diabetic patients that HbA_{1c} was directly correlated with MCP-1 ($R^2 = 0.728$, $P < 0.0001$, Fig. 1*E*; and $R^2 = 0.64$, $P < 0.01$, respectively), MDA ($R^2 = 0.56$, $P < 0.01$; and $R^2 = 0.53$, $P < 0.01$, respectively), and FPLPs ($R^2 = 0.54$, $P < 0.0001$; and $R^2 = 0.58$, $P < 0.01$, respectively), and was inversely associated with plasma vitamin E ($R^2 = -0.738$, $P < 0.0001$; and $R^2 = -0.38$, $P < 0.01$, respectively). Interestingly, circulating oxidant status was strongly associated with MCP-1 generation in the patients with microalbuminuria ($R^2 = -0.464$, $P = 0.005$ for vitamin E; $R^2 = 0.478$, $P = 0.005$ for MDA; $R^2 = 0.526$, $P < 0.001$ for FPLPs). Finally, MCP-1 showed a significant correlation with AER in the same group of patients ($R^2 = 0.547$, $P = 0.002$, Fig. 1*F*).

Effect of vitamin E on systemic MCP-1 biosynthesis in vivo

In the 15 microalbuminuric patients vitamin E treatment significantly raised plasma vitamin E (20 ± 3 vs. 39 ± 3 µmol/l, $P = 0.04$, Fig. 2*A*) to levels comparable to those observed in normoalbuminuric patients and in healthy control subjects at baseline. Interestingly, vitamin E treatment reduced MCP-1 biosynthesis (91 ± 10 vs. 63 ± 9 pg/ml, $P < 0.001$, Fig. 2*B*) to the level observed in normoalbuminuric patients at baseline. The effect of vitamin E on MCP-1 biosynthesis was also observed in the healthy volunteers treated with vitamin E (70 ± 8 vs. 52 ± 8, $P < 0.001$). Finally, AER was also significantly reduced by vitamin E in microalbuminuric patients (84 ± 34 vs. 58 ± 22 µg/min, $P = 0.03$, Fig. 2*C*), despite no changes in HbA_{1c} percentages.

Effect of vitamin E, insulin, and glucose on MCP-1 biosynthesis

Hyperglycemia has been reported to induce enhanced MCP-1 generation in nucleated cells (16). To further examine the relation between hyperglycemia and

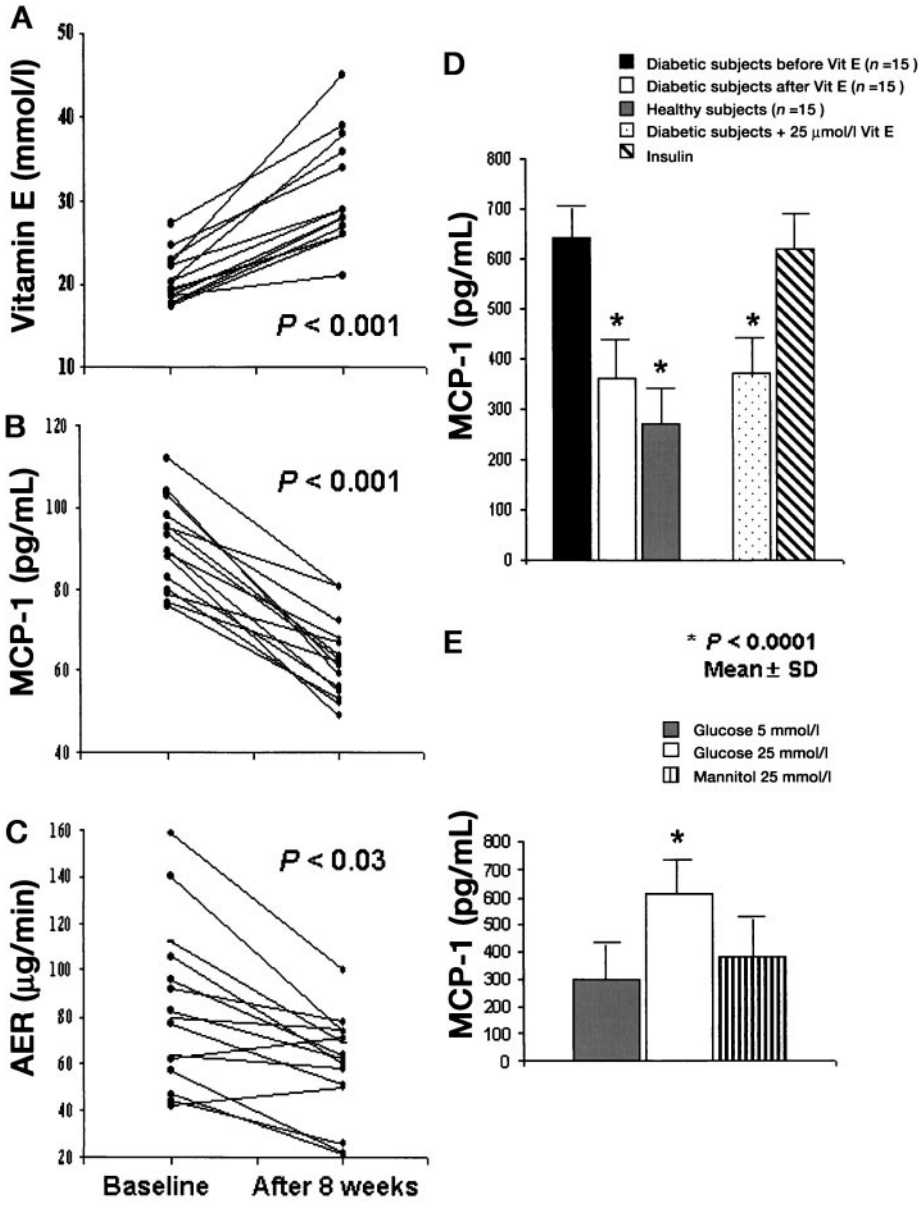

Figure 2—*Plasma levels of vitamin E (*A*) and MCP-1 (*B*), and AER (*C*) at baseline and after 8 weeks of high-dose vitamin E treatment in the diabetic patients with microalbuminuria and very poor glycemic control.* D*: The effect of serum from microalbuminuric diabetic patients on the spontaneous generation of MCP-1 in monocytes in vitro. Note inhibitory effect of vitamin E when administered both in vivo and in vitro. No effect of insulin was found.* E*: The effect of serum from healthy volunteers plus high glucose level compared with normal glucose level and mannitol in the generation of MCP-1 in myocytes in vitro. The bars and vertical lines represent mean ± SD values.*

monocyte activity in type 1 diabetes, monocytes from five healthy blood donors were evaluated for spontaneous MCP-1 generation after culturing for 24 h in a medium supplemented with either 20% serum from microalbuminuric diabetic patients before and after vitamin E therapy or 20% serum from healthy volunteers (Fig. 2*D*). Monocytes generated considerable levels of MCP-1 when cultured with serum collected from microalbuminuric diabetic patients at baseline (643 ± 42 pg/ml). Interestingly, significantly lower ($P < 0.0001$) MCP-1 generation was measured in monocytes cultured with serum collected from microalbuminuric diabetic patients after vitamin E treatment and from healthy subjects (363 ± 28 and 271 ± 21 pg/ml, respectively). The stronger stimulatory effect of serum collected at baseline from microalbuminuric diabetic patients on MCP-1 generation was blocked by the coincubation with 25 μmol/l vitamin E (643 ± 42 vs. 370 ± 22 pg/ml, $P < 0.0001$) but not with 6 μmol/l vitamin E (643 ± 42 vs. 607 ± 25 pg/ml, NS), thus confirming the critical role of high-dose vitamin E in controlling MCP-1 generation in monocytes. In contrast, enhanced MCP-1 generation was unaffected by incubation with insulin (643 ± 42 vs. 620 ± 35 pg/ml, NS), thus ruling out any effect of insulin on MCP-1 generation.

Moreover, in a second experiment (Fig. 2*E*), we observed more than a 100% increase in MCP-1 generation in monocytes cultured with serum from healthy volunteers plus high glucose (25 mmol/l) as compared with normal glucose (5 mmol/l) (611 ± 41 vs. 301 ± 13 pg/ml, $P < 0.0001$) or mannitol (611 ± 41 vs. 382 ± 31 pg/ml, $P < 0.0001$). Thus, this experiment supports the in vivo study by demonstrating that prolonged high glucose per se is responsible for the strong induction of MCP-1 biosynthesis in human healthy monocytes. Notably, a strong positive correlation between plasma MCP-1 in vivo and serum-induced monocyte MCP-1 generation in vitro was found in microalbuminuric diabetic patients both before ($R^2 = 0.401$, $P < 0.05$) and after vitamin E treatment ($R^2 = 0.438$, $P < 005$).

CONCLUSIONS — Persistent hyperglycemia is now well recognized as the major determinant of microvascular complications in diabetes (2). However, the precise mediators and biochemical pathways involved in this process are still unclear.

We found several important findings in this study. The first is that plasma MCP-1 was significantly increased in type 1 diabetic patients with early nephropathy when compared with matched patients without microvascular complications and healthy control subjects. Monocyte infiltration in the mesangium plays an important role in glomerular diseases (21) and is associated with fibroblast activation and increased extracellular matrix deposition in diabetic rats and diffuse glomerulosclerosis in patients with diabetic nephropathy (6,7). As MCP-1 is a potent chemoattractant for monocytes, it is of interest that increased glomerular expression of MCP-1 has been shown in sev-

eral glomerular diseases (20) as well as in the mesangium of rats with streptozotocin-induced diabetes (21). More recently, increased production of MCP-1 by blood mononuclear cells of patients with diabetes has been demonstrated (22).

Thus, we believe that our study presents several interesting and novel findings because, to the best of our knowledge, this is the first demonstration that MCP-1 is enhanced in vivo in adolescents and young adults with type 1 diabetes and is associated with early renal damage. Interestingly, in a recent study, Banba et al. (23) found that urine levels, but not serum levels, of MCP-1 increased in accordance with the extent of GHb and albuminuria. However, in this study only a small group of patients (9) had microalbuminuria and so could be correctly compared with our patients. Furthermore, eight of these nine patients had type 2 diabetes. Again, the age (mean ± SD) was 62.2 ± 10.4 years in the study from Banba et al. but only 18.5 ± 3.9 years in our study. Thus, we cannot exclude that different cellular sources may be involved in MCP-1 generation in type 1 diabetes with respect to type 2 diabetes, with a more systemic generation by circulating mononuclear cells in type 1 diabetes and a more limited renal production by mesangial cells in type 2 diabetes. Alternatively, different reactivity to hyperglycemia could be present in the inflammatory cells of diabetic patients with respect to age.

The second finding of this study is our observation of a correlation between MCP-1 and glycemic control. It has been demonstrated that high glucose concentration stimulates the expression of MCP-1 (16) and the formation of reactive oxygen species (ROS) (24), which may upregulate MCP-1 expression by activation of the transcription factor NF-κB (25). Furthermore, recent studies indicate that LDLox found in diabetic plasma have a potent biological ability to increase MCP-1 mRNA expression in nucleated cells (13,14). Notably, MCP-1 mRNA induced by lipoproteins from type 2 diabetic patients was significantly decreased by treatment with probucol, α-tocopherol, or deferoxamine, substances with known antioxidant activity (13). Our findings of the significant association between HbA_{1c}, plasma pro-oxidant status, and MCP-1 in diabetic patients with microalbuminuria support the hypothesis that persistent hyperglycemia can induce MCP-1 biosynthesis by increasing systemic oxidative stress. This hypothesis is further supported by our observation that MCP-1 production in monocytes in vitro is inhibited by vitamin E, but not insulin.

However, because diabetic patients without persistent microalbuminuria had normal MCP-1 levels, it is possible that hyperglycemia per se is necessary but not sufficient in determining increased MCP-1 expression in the setting of type 1 diabetes. Interestingly, plasma levels of vitamin E were significantly reduced in diabetic patients with poorer glycemic control and microalbuminuria, but not in diabetic subjects without microalbuminuria. All these observations suggest that moderate hyperglycemia is not a sufficient stimulus to induce vitamin E reduction and increased expression of MCP-1.

In contrast, we can hypothesize that prolonged hyperglycemia may lead to higher oxidative burden, consumption of endogenous antioxidant buffer (e.g., vitamin E), and overexpression of MCP-1. In agreement with this hypothesis, recent data have shown that antioxidant quercetin is able to inhibit expression of MCP-1 in glomerular cells (26) and that liver expression of MCP-1 was markedly reduced by vitamin E administration (27).

Reduced plasma vitamin E and increased circulating MCP-1 could be merely a secondary effect of diabetic nephropathy. However, this hypothesis is unlikely, because the direct role of vitamin E in MCP-1 generation is supported by the observation that in vivo generation of MCP-1 was reduced by administration of high-dose vitamin E—both in the 15 diabetic patients with microalbuminuria and in the 15 healthy volunteers. Again, one would speculate that most of the changes observed in diabetic patients after vitamin E are observed in response to treatment with insulin and improvement of glycemic control. However, this hypothesis is also unlikely, because in vitro MCP-1 generation in monocytes was exclusively downregulated by vitamin E, while insulin or changes in osmolar conditions failed to produce any effect. Moreover, glycemic control did not change in diabetic patients after vitamin E treatment, thus confirming that MCP-1 reduction after vitamin E was specifically due to vitamin E and not to improved glycemic control.

In our study, AER was significantly reduced after vitamin E administration. These results are in agreement with the recent study from Gaede et al. (28), in which a treatment with vitamin E (680 IU) plus vitamin C (1,250 mg) in type 2 diabetic patients with micro- or macroalbuminuria significantly lowers AER despite no changes in HbA_{1c}. In contrast, a subgroup analysis of 3,654 type 2 diabetic patients participating in the Heart Outcomes Prevention Evaluation (HOPE) study (29) demonstrated no renal effects in patients receiving low-dose (400 IU) vitamin E. However, some characteristics of the HOPE study may contribute to explain this apparent discrepancy. First, the daily dose of antioxidant administered in the HOPE study is significantly lower with respect to both our study and the study from Gaede et al. Thus, we can speculate that the simple administration of 400 IU of vitamin E may be not sufficient to restore the antioxidant supply to the level necessary to prevent the induction of inflammatory genes ultimately leading to renal damage and microalbuminuria. This hypothesis is also supported in our study by in vitro experiments, where the stronger stimulatory effect of serum collected at baseline from microalbuminuric diabetic patients on MCP-1 generation was blocked by the coincubation with 25 μmol/l vitamin E, but not with 6 μmol/l vitamin E, thus confirming that high doses of vitamin E are necessary for controlling MCP-1 generation in monocytes. Furthermore, because no information on compliance and plasma values of vitamin E has been reported in the HOPE study, we cannot exclude that the failure of vitamin E in reducing AER in this study may be due, at least in part, to nonadequate vitamin E bioavailability.

In conclusion, our study supports the hypothesis that upregulation of MCP-1 gene expression by persistent hyperglycemia in type 1 diabetic patients results in the recruitment of monocytes into the kidney, possibly contributing to the development of diabetic nephropathy. Moreover, these results suggest that the causative role of poor glycemic control in diabetic nephropathy is mediated by increased oxidative stress and reduced vitamin E plasma level. These findings are potentially important from a fundamental stand point because they indicate a pathogenetic role for MCP-1 in the evolution of diabetic microvascular complications. From a practical perspective, these results raise the possibility that vitamin E may provide a novel form of therapy for pre-

vention of microvascular complications in type 1 diabetic patients, in whom an acceptable glycemic control is difficult to achieve despite appropriate insulin treatment.

Acknowledgments— This research was supported by the Italian Ministry for Research (MURST) Funds, the local Juvenile Diabetes Foundation (AGD Abruzzo), and by an educational grant from Pharmacia, Italy.

We acknowledge the editorial assistance of Antonella Bascelli. We also thank Doriana D'Alimonte D'Attilio (AGD Abruzzo) for her invaluable help in the care of children with diabetes.

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