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Elevated Plasma Asymmetric Dimethyl-L-Arginine Levels Are Linked to Endothelial Progenitor Cell Depletion and Carotid Atherosclerosis in Rheumatoid Arthritis

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Objective. **Similarities between rheumatoid arthritis (RA) and atherosclerosis include endothelial dysfunction (an antecedent of plaque formation) and depletion of circulating bone marrow–derived endothelial progenitor cells. This study was undertaken to test the hypothesis that endothelial progenitor cell depletion and subclinical atherosclerosis in RA may be related to accumulation of an endogenous inhibitor of nitric oxide (NO) synthesis, asymmetric dimethyl-L-arginine.**

Methods. **We studied 30 patients with active RA and 20 age- and sex-matched healthy controls. Exclusion criteria were clinically evident atherosclerosis, traditional risk factors, hyperhomocysteinemia, and renal dysfunction. The blood endothelial progenitor cell count was assayed by flow cytometry and expressed as a percentage of lymphocytes. Plasma L-arginine, asymmetric dimethyl-L-arginine, and symmetric dimethyl-L-arginine were measured with liquid chromatography–mass spectrometry. Mean carotid intima-media thickness (IMT) was assessed by B-mode ultrasound.**

Results. **In RA patients, we found elevated levels of asymmetric dimethyl-L-arginine (mean ± SD 0.49 ± 0.07 μmoles/liter versus 0.40 ± 0.07 μmoles/liter in controls; $P < 0.001$), a depressed endothelial progenitor cell count (0.039 ± 0.025% versus 0.063 ± 0.035%; $P < 0.05$), and increased IMT (0.65 ± 0.13 mm versus 0.55 ± 0.10 mm; $P < 0.01$), with no differences in levels of L-arginine or symmetric dimethyl-L-arginine. The endothelial progenitor cell count was inversely correlated with the level of asymmetric dimethyl-L-arginine. IMT was positively related to the ratio of asymmetric dimethyl-L-arginine to L-arginine and negatively related to the endothelial progenitor cell count, in univariate and multivariate analyses.**

Conclusion. **Plasma asymmetric dimethyl-L-arginine levels are elevated in RA patients free of cardiovascular disease or risk factors. Asymmetric dimethyl-L-arginine accumulation may contribute to endothelial progenitor cell depletion via depressed NO-dependent endothelial progenitor cell mobilization and/or survival, with consequent impairment of endothelial progenitor cell–mediated endothelial repair, which can promote atherogenesis in RA.**

Rheumatoid arthritis (RA), a disease affecting ~1% of the general adult population, is associated with accelerated atherogenesis and excessive cardiovascular morbidity and mortality (1–4). In patients with RA, elevated cardiovascular mortality (2–4) and increased carotid intima-media thickness (IMT) (5–7), a noninvasive index of subclinical atherosclerosis, cannot be fully explained by traditional risk factors and are attributed to inflammatory activation, disease duration, and disease progression.

Numerous analogies between RA and atherosclerosis have been reported, including macrophage, T cell, and mast cell activation, altered Th1/Th2 balance, ele-

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vated levels of circulating acute-phase reactants and adhesion molecules, increased generation of endothelins, oxidative stress, and activation of neoangiogenesis (1,8,9). This set of similarities has been extended to include endothelial dysfunction, which has been demonstrated in patients with early (10) and long-term (11) RA. Endothelial dysfunction occurred in the absence of manifest cardiovascular disease and was unrelated to traditional atherosclerotic risk factors.

Endothelial dysfunction has also been found in a highly selected subgroup of young to middle-aged patients with RA with low disease activity and no overt atherosclerotic disease or classic risk factors (12). It is noteworthy that endothelial dysfunction has been improved by treatment with various disease-modifying antirheumatic drugs (DMARDs) in patients with newly diagnosed RA (10), as well as after addition of an antagonist of tumor necrosis factor α (TNFα) to the treatment regimen of RA patients with high disease activity despite combination therapy with methotrexate and prednisone (13). However, endothelial dysfunction has also been reported in RA patients with low disease activity controlled by long-term DMARD therapy (11,12).

It has recently been shown that RA patients exhibit depressed levels of circulating bone marrow–derived endothelial progenitor cells (14,15), a subset of mononuclear white blood cells participating in neovascularization of ischemic tissue (16) and reendothelization of injured arterial walls (17). Following the detection of endothelial progenitor cells in the synovial tissue of RA patients (18), it was proposed that enhanced recruitment of endothelial progenitor cells from the peripheral blood into the inflamed and hypoxic synovium may result in the depletion of circulating endothelial progenitor cells, which might translate into augmented cardiovascular risk (14,19). This cardiovascular risk augmentation might be due to impaired formation of new blood vessels by peripheral recruitment of endothelial progenitor cells (vasculogenesis) (14,19) and/or impaired ongoing endothelial repair (17), with consequent acceleration of atherogenesis and plaque destabilization.

Previously, it has been demonstrated that the endothelial progenitor cell count in peripheral blood is depressed in patients with angiographically proven coronary artery disease (CAD) (20). Moreover, in individuals without clinical evidence of atherosclerosis yet with conventional atherosclerotic risk factors, the endothelial progenitor cell count has been found to be inversely correlated with the magnitude of endothelial dysfunction (21). Endothelial dysfunction (an antecedent of atherosclerotic plaque development) (22), endothelial progenitor cell depletion (23,24), and elevated levels of a circulating endogenous inhibitor of nitric oxide (NO) synthesis, N^G,N^G-dimethylarginine (asymmetric dimethyl-L-arginine) (25), are independent predictors of future cardiovascular events in patients with CAD.

The L-arginine–NO pathway participates in endothelial progenitor cell mobilization from the bone marrow (26–28), and an inverse correlation between the level of plasma asymmetric dimethyl-L-arginine and the blood endothelial progenitor cell count has recently been described (29). Our aim, therefore, was to test the hypothesis that abnormal levels of plasma asymmetric dimethyl-L-arginine may be linked to endothelial progenitor cell depletion and subclinical atherosclerosis in RA.

PATIENTS AND METHODS

Patients. Thirty patients (25 women and 5 men, with a mean ± SD age of 47 ± 12 years and a mean ± SD disease duration of 5.1 ± 4.4 years) with active RA despite long-term DMARD therapy were studied. All patients had a disease duration of ≥1 year and had been on an unchanged DMARD-based therapeutic regimen for ≥3 months. RA was diagnosed according to the revised 1987 American College of Rheumatology (formerly, the American Rheumatism Association) criteria (30). Active RA was defined as a Disease Activity Score in 28 joints (DAS28) (31) ≥3.2, similar to the guidelines used by Grisar et al (14), who were the first investigators to report endothelial progenitor cell depletion in RA.

Patients were excluded if they had a DAS28 <3.2, were receiving steroid therapy at the time of the study, were >60 years or <25 years of age, had a history of or current clinical evidence of cardiovascular disease, smoked at the time of the study, had ceased smoking <2 years before the beginning of the study, or had arterial hypertension (blood pressure ≥140/90 mm Hg or a history of receiving antihypertensive therapy). Additional exclusion criteria included a low-density lipoprotein cholesterol level >4.14 mmoles/liter, a triglyceride level >2.29 mmoles/liter, an estimated glomerular filtration rate (GFR) <60 ml/minute/1.73 m^2 of body surface area (calculated using the modified Modification of Diet in Renal Disease study [32] equation), any history of kidney damage irrespective of the GFR, a fasting homocysteine level ≥12 μmoles/liter, endocrinologic disorders, including diabetes mellitus or impaired fasting glucose (>5.5 mmoles/liter in venous plasma), a family history of premature CAD (occurring at ages <55 years in men and <65 years in women) in first-degree relatives, abnormalities of thyroid or liver function, neoplastic diseases, and any infections within the 2 months prior to study entry. Patients with severe extraarticular manifestations of RA, a factor strongly linked to prognosis (33), were also excluded.

None of the patients were receiving statins, which have been shown to affect the endothelial progenitor cell count (34), or any other cardiovascular medications. All patients were receiving classic nonsteroidal antiinflammatory drugs and

Table 1. Characteristics of the RA patients and healthy controls*

Characteristic	RA patients (n = 30)	Controls (n = 20)
Age, years	47 ± 12	45 ± 10
Sex, no. (%) female	25 (83)	17 (85)
BMI, kg/m^2	25.2 ± 4.3	25.0 ± 2.1
Disease duration, years	5.1 ± 4.4	NA
DAS28	5.9 ± 1.2	NA
RF positivity, no. (%)	21 (70)	NA
MTX, no. (%)	18 (60)	NA
MTX plus other DMARD, no. (%)	6 (20)	NA
hsCRP, median (IQR) mg/liter	12.0 (1.6–37.4)†	0.8 (0.6–1.0)
Homocysteine, μmoles/liter	9.3 ± 3.8	9.5 ± 1.6
Mean arterial pressure, mm Hg	88 ± 5	89 ± 6
Estimated GFR, ml/minute/1.73 m^2	104 ± 27	108 ± 13
LDL cholesterol, mmoles/liter	2.9 ± 0.9	3.2 ± 0.4
HDL cholesterol, mmoles/liter	1.0 ± 0.3†	1.3 ± 0.3
Triglycerides, mmoles/liter	1.3 ± 0.7	1.1 ± 0.3
Glucose, mmoles/liter	4.8 ± 0.4	4.9 ± 0.4
VEGF, median (IQR) pg/ml	47 (29–79)†	14 (8–20)
IMT, mm	0.65 ± 0.13‡	0.55 ± 0.10
Subjects with carotid plaques, no. (%)	8 (27)§	0 (0)

* Except where indicated otherwise, values are the mean ± SD. RA = rheumatoid arthritis; BMI = body mass index; NA = not applicable; DAS28 = Disease Activity Score in 28 joints; RF = rheumatoid factor; MTX = methotrexate; DMARD = disease-modifying antirheumatic drug; hsCRP = high-sensitivity C-reactive protein; IQR = interquartile range; GFR = glomerular filtration rate; LDL = low-density lipoprotein; HDL = high-density lipoprotein; VEGF = vascular endothelial growth factor; IMT = intima-media thickness.
† $P < 0.001$ versus controls.
‡ $P < 0.01$ versus controls.
§ $P = 0.015$ versus controls.

DMARDs at the time of study entry; for 80% of patients, the DMARD regimen was based on methotrexate (MTX), administered alone or in combination with another DMARD (Table 1). All patients who received MTX also received regular folic acid supplementation. None of the patients were receiving TNFα antagonists, which are believed to increase the endothelial progenitor cell count (14), or selective cyclooxygenase 2 inhibitors.

In addition to RA patients, we studied 20 age- and sex-matched healthy controls, who were also subject to the exclusion criteria listed above. The study was performed in accordance with the Declaration of Helsinki, and approval was obtained from the bioethics committee of Jagiellonian University.

Flow cytometric analysis. After patients and control subjects fasted overnight, blood sampling was performed for routine and extended biochemical assays. At this time, blood samples for fluorescence-activated cell sorting (FACS) were obtained from an antecubital vein. The procedure was performed <60 minutes after venipuncture, according to the method described by Vasa et al (20). Briefly, 100 μl of blood was incubated in the dark with mouse monoclonal antibodies against human vascular endothelial growth factor (VEGF) receptor 2/kinase insert domain receptor (KDR; Sigma, St. Louis, MO), followed by rabbit fluorescein isothiocyanate (FITC)–labeled secondary antibodies (Dako, Carpinteria, CA) and phycoerythrin (PE)–conjugated mouse monoclonal antibodies against human CD34 (Becton Dickinson, Mountain View, CA). Control blood samples were incubated with mouse isotype-matched antibodies (FITC-conjugated IgG1 and PE-conjugated IgG2a) (γ_1/γ_{2a} Simultest; Becton Dickinson). After cell lysis, FACS was performed, including 60,000–100,000 events acquired within the lymphocyte gate (FACScan; Becton Dickinson, Heidelberg, Germany). Endothelial progenitor cells were defined as CD34+,KDR+ cells, and their number was expressed as a percentage of cells within the lymphocyte gate (20,23).

Biochemical assays. For measurement of L-arginine and its dimethylated analogs (asymmetric dimethyl-L-arginine and symmetric dimethyl-L-arginine) in plasma, we used a recently described and validated liquid chromatography tandem mass spectrometry method with an isotope-labeled internal standard (35). Precision and accuracy tests for the asymmetric dimethyl-L-arginine assay revealed intraday and interday relative SDs of 5.5% and 7.7%, respectively, whereas respective inaccuracies were 2.6% and 8.0%. Relative SDs and inaccuracies for L-arginine and symmetric dimethyl-L-arginine were <5.2%.

High-sensitivity C-reactive protein (hsCRP) and homocysteine concentrations were assessed using commercially available chemiluminescence immunoassay systems (Immulite 1000 and Immulite 2000; DPC, Flanders, NJ). Levels of VEGF were measured using an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). The lower detection limit was 5 pg/ml, and intraassay and interassay relative SDs were 6.7% and 8.8%, respectively.

Ultrasonography of the common carotid arteries. IMT of the common carotid artery was estimated by B-mode ultrasonography using an instrument equipped with a 4.0–10.0 MHz vascular transducer (Vivid 7; GE Medical Systems, Piscataway, NJ). Ultrasonography was performed by an investigator (TR) who was unaware of subjects' clinical backgrounds. The final IMT value for each patient was the average of 12 measurements made at end diastole. These measurements corresponded to the distances between the lumen–intima interface and media–adventitia interface on both sides at the far (distal to the skin) and near (proximal to the skin) arterial wall at 3 points within a 1.5-cm segment immediately caudal to the carotid bulb. Wall thickness was measured outside atherosclerotic plaques (if present), defined as encroachments into the vessel lumen with a distinct area ≥50% thicker than the surrounding wall (6,7). The intraobserver and interobserver relative SDs of IMT measurements were 4.9% and 6.4%, respectively, as assessed in a group of 20 subjects examined in our laboratory.

Statistical analysis. Data are reported as the mean ± SD unless indicated otherwise. Intergroup comparisons of individual continuous variables between RA patients and healthy controls were performed using Student's 2-tailed *t*-test. Then, RA patients were divided into 2 groups, those with carotid plaques and those without carotid plaques. Normally distributed continuous variables were compared between

healthy controls, RA patients without carotid plaques, and RA patients with carotid plaques by one-way analysis of variance (ANOVA). *P* values less than 0.05 were considered significant. If one-way ANOVA results were statistically significant, post hoc intergroup comparisons were performed using Tukey's honest significant difference test. The accordance with a normal distribution was tested by the Kolmogorov-Smirnov test, and uniformity of variance (homoscedasticity) was tested by Levene's test. Logarithmic transformation was applied to the endothelial progenitor cell count in order to obtain a normal distribution. The ratio of asymmetric dimethyl-L-arginine to L-arginine was log-transformed to eliminate heteroscedasticity. Due to the lack of homogeneity of variances, levels of hsCRP and VEGF were compared using the Mann-Whitney U test and Kruskal-Wallis ANOVA on ranks and reported as the median and interquartile range. Intergroup differences in categorical variables were assessed by the chi-square test or Fisher's exact test.

In order to identify correlates of IMT and log(endothelial progenitor cell count), Pearson's univariate correlation coefficients (r) were calculated for RA patients as a whole and for healthy controls considered separately. In order to detect independent determinants of IMT, forward stepwise multiple linear regression was performed. Only variables for which the *P* value in a univariate analysis was <0.15 were included in the multiple regression. Coefficients of multiple determination (R^2) were determined, along with standard mean regression coefficients (β) and the SEM for individual variables entering the final regression equation.

RESULTS

Clinical characteristics of the RA patients and healthy controls. Compared with healthy controls, RA patients exhibited higher hsCRP and VEGF levels, elevated mean IMT, and lower high-density lipoprotein (HDL) cholesterol levels. Carotid plaques were detected in 8 of 30 RA patients (27%) and in none of the healthy controls ($P = 0.015$) (Table 1).

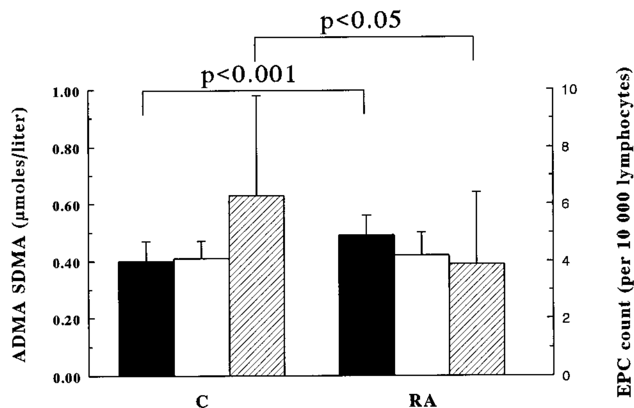

Figure 1. Levels of plasma asymmetric dimethyl-L-arginine (ADMA), endothelial progenitor cell (EPC) counts, and levels of plasma symmetric dimethyl-L-arginine (SDMA) in patients with rheumatoid arthritis (RA) and in healthy controls (C). In patients with RA, levels of plasma ADMA (solid bars) were elevated and EPC counts (hatched bars) were depressed compared with healthy controls. No significant differences in levels of plasma SDMA (open bars) were found. Values are the mean and SD.

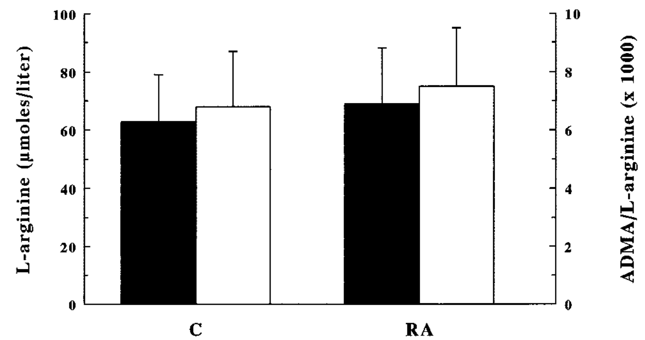

Figure 2. Levels of plasma L-arginine and ratio of ADMA to L-arginine in patients with RA and in healthy controls. No significant differences in levels of plasma L-arginine (solid bars) or the ratio of ADMA to L-arginine (open bars) between healthy controls and RA patients were found. Values are the mean and SD. See Figure 1 for definitions.

Endothelial progenitor cell count and L-arginine and its dimethylated derivatives in RA patients and healthy controls. Compared with healthy controls, RA patients exhibited depressed endothelial progenitor cell counts (mean $\pm$ SD $0.039 \pm 0.025\%$ versus $0.063 \pm 0.035\%$ in controls; $P < 0.05$) and elevated plasma asymmetric dimethyl-L-arginine levels (0.49 ± 0.07 µmoles/liter versus 0.40 ± 0.07 µmoles/liter; $P < 0.001$) (Figure 1). No significant intergroup differences in plasma concentrations of symmetric dimethyl-L-arginine (0.42 ± 0.08 µmoles/liter versus 0.41 ± 0.06 µmoles/liter; $P = 0.81$) (Figure 1), L-arginine (69 ± 19 µmoles/liter versus 63 ± 16 µmoles/liter; $P = 0.17$), or the ratio of asymmetric dimethyl-L-arginine to L-arginine (0.0075 ± 0.0020 versus 0.0068 ± 0.0019; $P = 0.20$) (Figure 2) were found.

Intergroup comparisons of asymmetric dimethyl-L-arginine level, ratio of asymmetric dimethyl-L-arginine to L-arginine, and blood endothelial progenitor cell count in relation to the presence or absence of carotid plaques. Asymmetric dimethyl-L-arginine levels were elevated to a comparable degree in RA patients without carotid plaques and RA patients with carotid plaques, compared with healthy controls ($P < 0.001$ by ANOVA) (Table 2). RA patients with carotid plaques exhibited a significantly lower endothelial progenitor cell count, a higher ratio of asymmetric dimethyl-L-arginine to L-arginine, and increased IMT, compared with either RA patients without carotid plaques or

Table 2. IMT, endothelial progenitor cell count, and plasma levels of L-arginine, asymmetric dimethyl-L-arginine, and symmetric dimethyl-L-arginine in healthy controls and in RA patients without and those with carotid plaques*

	Controls (n = 20)	RA patients without carotid plaques (n = 22)	RA patients with carotid plaques (n = 8)
IMT, mm	0.55 ± 0.10	0.61 ± 0.12	0.74 ± 0.11†
Endothelial progenitor cells, % of lymphocytes	0.063 ± 0.031	0.045 ± 0.032	0.020 ± 0.013‡
L-arginine, μmoles/liter	63 ± 16	73 ± 18	61 ± 19
Asymmetric dimethyl-L-arginine, μmoles/liter	0.40 ± 0.07	0.48 ± 0.07§	0.52 ± 0.08¶
Symmetric dimethyl-L-arginine, μmoles/liter	0.41 ± 0.06	0.43 ± 0.08	0.38 ± 0.09
Ratio of asymmetric dimethyl-L-arginine to L-arginine	0.0068 ± 0.0019	0.0069 ± 0.0013	0.0092 ± 0.0028‡

* Values are the mean ± SD. All P values were determined by Tukey's test. See Table 1 for definitions.
† $P < 0.05$ versus RA patients without carotid plaques; $P < 0.01$ versus controls.
‡ $P < 0.05$ versus RA patients without carotid plaques and versus controls.
§ $P < 0.01$ versus controls.
¶ $P < 0.001$ versus controls.

healthy controls ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively, by ANOVA) (Table 2).

Due to the lack of significant differences in other variables between RA patients without carotid plaques and RA patients with carotid plaques (Table 3), ANOVA-based comparisons were performed to confirm the above-noted differences between the group of RA patients as a whole and healthy controls in levels of hsCRP, HDL cholesterol, and VEGF.

Correlates of IMT and endothelial progenitor cell count. In RA patients, IMT correlated with the ratio of asymmetric dimethyl-L-arginine to L-arginine (r = 0.48, $P = 0.007$), age (r = 0.43, $P = 0.02$), asymmetric dimethyl-L-arginine levels (r = 0.38, $P = 0.04$) (Figure 3), and log(endothelial progenitor cell count) (r = −0.38, $P = 0.04$). Log(endothelial progenitor cell count) correlated inversely with asymmetric dimethyl-L-arginine levels (r = −0.41, $P = 0.02$) (Figure 4).

Forward stepwise multiple linear regression identified the ratio of asymmetric dimethyl-L-arginine to L-arginine ($\beta = 0.41 \pm 0.15$, $P = 0.01$) and log(endothelial progenitor cell) ($\beta = -0.32 \pm 0.15$, $P = 0.04$) as independent predictors of IMT in RA patients ($R^2 = 0.42$, $P = 0.002$; n = 30). When asymmetric dimethyl-L-arginine instead of the ratio of asymmetric dimethyl-L-arginine to L-arginine was forced into the regression

Table 3. Characteristics of RA patients without and those with carotid plaques*

Characteristic	RA patients without carotid plaques (n = 22)	RA patients with carotid plaques (n = 8)
Age, years	45 ± 11	52 ± 14
Sex, no. (%) female	18 (81.8)	7 (87.5)
BMI, kg/m^2	25.0 ± 4.7	26.1 ± 3.2
Disease duration, years	5.2 ± 4.5	4.8 ± 4.3
DAS28	6.0 ± 1.4	5.6 ± 0.5
RF positivity, no. (%)	14 (63.6)	7 (87.5)
MTX, no. (%)	14 (63.6)	4 (50)
MTX plus other DMARD, no. (%)	2 (9.1)	4 (50)
hsCRP, median (IQR) mg/liter	7.5 (1.7–45.1)†	23.5 (1.2–37.9)‡
Homocysteine, μmoles/liter	9.7 ± 4.0	8.2 ± 3.0
Estimated GFR, ml/minute/1.73 m^2	101 ± 22	116 ± 39
Leukocytes, 10^3/μl	7.1 ± 1.9	7.2 ± 2.5
LDL cholesterol, mmoles/liter	2.7 ± 0.9	3.3 ± 0.5
HDL cholesterol, mmoles/liter	1.0 ± 0.3†	0.9 ± 0.3†
Glucose, mmoles/liter	4.7 ± 0.4	4.9 ± 0.5
Mean arterial pressure, mm Hg	88 ± 5	87 ± 6
VEGF, median (IQR) pg/ml	49 (29–82)†	47 (27–61)†

* Except where indicated otherwise, values are the mean ± SD. See Table 1 for definitions.
† $P < 0.001$ versus controls.
‡ $P < 0.01$ versus controls.

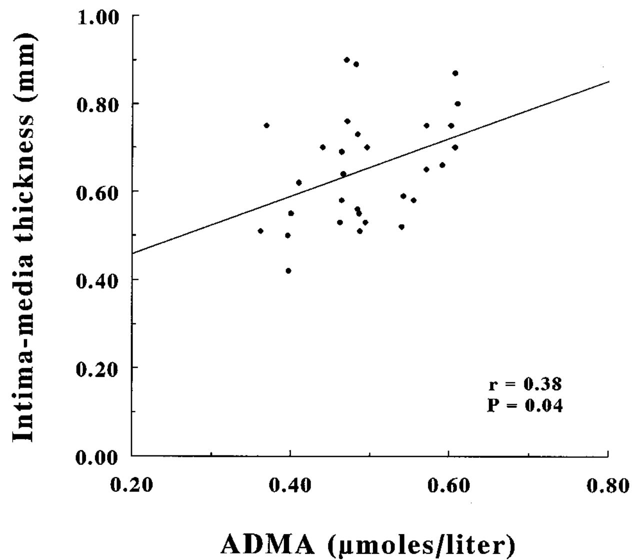

Figure 3. Correlation between plasma ADMA levels and mean intima-media thickness of the common carotid arteries in RA patients. See Figure 1 for definitions.

model describing IMT, R^2 (representing the proportion of IMT variability accounted for by the regression equation) was lower ($R^2 = 0.33$, $P = 0.01$; n = 30), and the final set of variables was as follows: age ($\beta = 0.34$ [SEM 0.13], $P = 0.009$), log(endothelial progenitor cell count) ($\beta = -0.25$ [SEM 0.12], $P = 0.04$), and asymmetric dimethyl-L-arginine ($\beta = 0.25$ [SEM 0.12], $P = 0.045$).

In healthy controls, IMT and endothelial progenitor cell count were not significantly correlated with each other or with other variables. Trends toward correlations between IMT and age (r = 0.39, $P = 0.09$) and between IMT and asymmetric dimethyl-L-arginine (r = 0.36, $P = 0.12$) were found, but they were not statistically significant. Limitation of analyses to women, who constituted 83% of the RA patients and 85% of the controls, produced results similar to those presented above (data not shown).

DISCUSSION

Our results indicate that RA is associated with increased asymmetric dimethyl-L-arginine levels and a depressed endothelial progenitor cell count, both of which may contribute to accelerated early atherogenesis. These phenomena appear unrelated to coexistent abnormalities, since RA patients and controls were age matched, and patients were excluded from the study if they had conditions previously reported to be associated with elevated asymmetric dimethyl-L-arginine levels and/or a lowered endothelial progenitor cell count (20,23,24,36,37). Asymmetric dimethyl-L-arginine levels

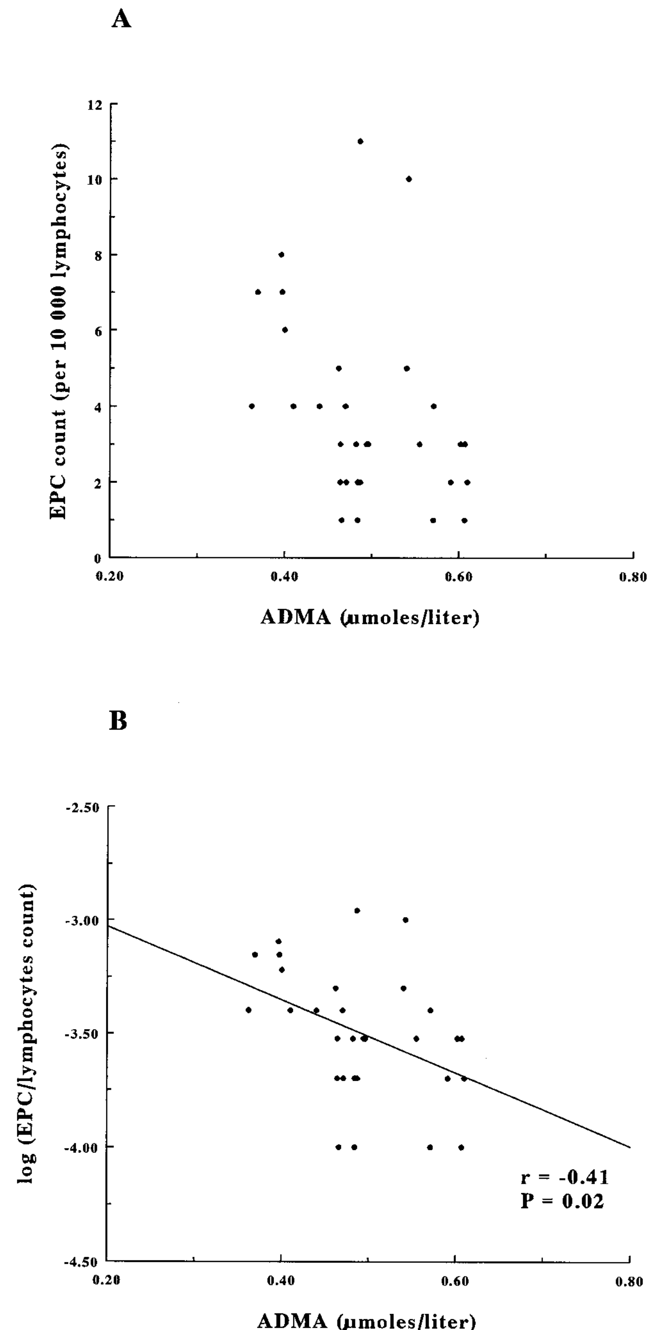

Figure 4. Correlation between plasma ADMA levels and EPC count in RA patients. **A,** Nontransformed data points. **B,** Regression line and data points depicting the log-transformed ratio of EPC to lymphocyte count as a function of the plasma ADMA level. See Figure 1 for definitions.

were elevated to a comparable degree in RA patients with carotid plaques and RA patients without carotid plaques; this suggests that RA itself is associated with elevated asymmetric dimethyl-L-arginine levels.

Eighty percent of the RA patients in the present study received MTX, a drug known to interfere with folate metabolism via inhibition of dihydrofolate reductase. MTX-induced 5-methyltetrahydrofolate depletion, with consequently depressed remethylation of homocysteine to methionine (38), might theoretically impair S-adenosylmethionine–dependent methylation reactions, including asymmetric dimethyl-L-arginine formation, which could lower circulating asymmetric dimethyl-L-arginine levels. However, we observed elevated levels of asymmetric dimethyl-L-arginine in RA patients. In addition, MTX-treated patients received folate supplements, and homocysteine levels were similar in patients with RA and control subjects.

Several mechanisms of asymmetric dimethyl-L-arginine elevation in RA can be proposed. First, decreases in the activity of dimethylarginine dimethylaminohydrolase, a key enzyme governing asymmetric dimethyl-L-arginine (but not symmetric dimethyl-L-arginine) degradation, may increase asymmetric dimethyl-L-arginine levels. Asymmetric dimethyl-L-arginine is liberated during the breakdown of proteins containing methylated arginine residues (36). The activity of dimethylarginine dimethylaminohydrolase is negatively regulated by TNFα (39), oxidative stress (39), and S-nitrosylation (40). It has been estimated that dimethylarginine dimethylaminohydrolase catalyzes degradation of >80% of the asymmetric dimethyl-L-arginine generated daily (36). Since increases in TNFα (1), reactive oxygen species (41), and 3-nitrotyrosine (42) have been reported in RA, we hypothesize that selective increases in asymmetric dimethyl-L-arginine levels in RA could result from lowered dimethylarginine dimethylaminohydrolase activity.

A second possibility is that oxidative stress associated with RA might increase the formation of asymmetric dimethyl-L-arginine via augmented expression of protein arginine type I *N*-methyltransferases (43). The substrates of these arginine type I *N*-methyltransferases are protein side chain arginine guanidine groups.

Third, in the inflamed synovium of RA patients, increased proliferation and potentiated apoptosis of vascular endothelial cells have been reported (44). Cultured endothelial cells release more asymmetric dimethyl-L-arginine than symmetric dimethyl-L-arginine (45). Therefore, increased endothelial cell turnover in RA, with consequent liberation of free asymmetric dimethyl-L-arginine during protein catabolism, could contribute to elevated levels of asymmetric dimethyl-L-arginine.

Fourth, hypoxia within the inflamed synovium in RA is a well-recognized phenomenon (44). Hypoxia is known to decrease expression of dimethylarginine dimethylaminohydrolase (46). A decrease in dimethylarginine dimethylaminohydrolase activity may increase asymmetric dimethyl-L-arginine levels, as discussed above.

Last, a tendency toward insulin resistance has been observed in RA patients (1), and a positive association between plasma asymmetric dimethyl-L-arginine levels, whole-body protein turnover rate, and insulin resistance has been described in healthy subjects with various levels of insulin sensitivity (47). This phenomenon is hypothetically linked to enhanced asymmetric dimethyl-L-arginine release during accelerated cleavage of protein molecules. The significantly lower levels of HDL cholesterol in the RA patients in this study, as compared with controls, might suggest the presence of insulin resistance–associated dyslipidemia. However, in the study by Marliss et al (47), both asymmetric dimethyl-L-arginine and symmetric dimethyl-L-arginine were correlated with indices of glucose and protein metabolism, whereas in the present study only selective increases in asymmetric dimethyl-L-arginine were observed.

Following an early study by Miyazaki et al (37) of 116 subjects without clinical evidence of atherosclerosis, associations between asymmetric dimethyl-L-arginine and carotid IMT were also shown in end-stage renal disease (48). This suggests a role of asymmetric dimethyl-L-arginine in atherogenesis over a wide range of asymmetric dimethyl-L-arginine levels measured in clinical conditions. Despite the fact that plasma asymmetric dimethyl-L-arginine concentrations are low as compared with L-arginine levels, and the Michaelis-Menten constant (K_m) of endothelial NO synthase for L-arginine is ~2.9 mmoles/liter, proatherogenic effects of asymmetric dimethyl-L-arginine have been suggested on the basis of experimental and clinical data, as described below.

First, intracellular asymmetric dimethyl-L-arginine concentrations appear 8–12-fold higher than extracellular levels (45), and the K_m of dimethylarginine dimethylaminohydrolase for asymmetric dimethyl-L-arginine is as high as 180 μmoles/liter, which may reflect local elevations of intracellular asymmetric dimethyl-L-arginine (36). Second, an ability of exogenous asymmet-

ric dimethyl-L-arginine to produce biologic effects attributable to depressed NO synthesis has been observed at levels of asymmetric dimethyl-L-arginine comparable with those measured in pathophysiologic conditions in vivo (36). Third, asymmetric dimethyl-L-arginine is capable of initiating processes involved in early phases of atherogenesis, namely increased endothelial secretion of monocyte chemoattractant protein 1 (45), and accelerated senescence of endothelial cells, the latter occurring via interference with telomerase activity (49). Importantly, blockade (50) or overexpression (51) of dimethylarginine dimethylaminohydrolase resulted in measurable effects related to NO deficiency or excess, respectively, which suggests a continuous regulation of NO bioavailability by asymmetric dimethyl-L-arginine and dimethylarginine dimethylaminohydrolase. Finally, asymmetric dimethyl-L-arginine has been identified as an independent predictor of adverse cardiovascular events in end-stage renal failure (52) and CAD (25).

The limited number of patients in the present study allows only cautious conclusions. However, a correlation between lowered endothelial progenitor cell count and elevated asymmetric dimethyl-L-arginine levels in RA, and an independent association of the ratio of asymmetric dimethyl-L-arginine to L-arginine with IMT, provide evidence that endothelial progenitor cell–asymmetric dimethyl-L-arginine interactions may be a novel hypothetical mechanism of proatherogenic asymmetric dimethyl-L-arginine activity. In addition, RA patients with carotid plaques exhibited both an elevated ratio of asymmetric dimethyl-L-arginine to L-arginine and a lower blood endothelial progenitor cell count, as compared with RA patients without plaques or with healthy controls. These results are consistent with the findings of a recent study by Thum et al (29), who demonstrated an ability of exogenous asymmetric dimethyl-L-arginine to impair differentiation and function of isolated endothelial progenitor cells as well as a negative correlation between the endothelial progenitor cell count and asymmetric dimethyl-L-arginine levels in patients with CAD. Therefore, it might be hypothesized that some proatherogenic effects of asymmetric dimethyl-L-arginine could be mediated by its interference with the endothelial progenitor cell count and/or function.

Mobilization of endothelial progenitor cells from the bone marrow in response to VEGF (27), statins (28), myocardial ischemia (28), and estradiol (26) is dependent on NO formation. Augmented release of VEGF from the inflamed and hypoxic synovium is responsible for high plasma VEGF levels in patients with RA, especially in patients with high disease activity (53), such as those in the present study. It has been proposed that VEGF activates endothelial cell NO synthase in vascular cells of the bone marrow stroma, by an Akt-dependent mechanism, with consequent matrix metalloproteinase 9 activation/overexpression, which triggers endothelial progenitor cell mobilization (27,54). Thus, asymmetric dimethyl-L-arginine–mediated impairment of NO activity in the bone marrow can interfere with this sequence of events, with subsequent impairment of endothelial progenitor cell mobilization, which may contribute to blood endothelial progenitor cell depletion and explain low circulating endothelial progenitor cell counts despite elevated levels of VEGF.

Admittedly, we have not studied the functional properties of endothelial progenitor cells, e.g., their ability to differentiate and incorporate into endothelial tube-like structures, NO-dependent processes (55,56) that can also be inhibited by asymmetric dimethyl-L-arginine (29). It could be proposed that functional endothelial progenitor cell insufficiency due to lowered NO formation might offer an alternative mechanism of proatherogenic endothelial progenitor cell–asymmetric dimethyl-L-arginine interactions, irrespective of the circulating endothelial progenitor cell count. These 2 possible mechanisms of endothelial progenitor cell–asymmetric dimethyl-L-arginine interactions, which are not mutually exclusive, both assume the participation of endothelial progenitor cells in ongoing endothelial repair. Indeed, integration of bone marrow–derived endothelial progenitor cells into the endothelial lining of the coronary arteries of patients who have received heart transplants has been shown (57), thus confirming previous animal data on endothelial progenitor cell contribution to reendothelization after carotid balloon injury (17).

According to a hypothesis set forth by Vasa et al (20), endothelial progenitor cell depletion can promote atherogenesis through an imbalance between continuous endothelial cell renewal and injury, with subsequent endothelial dysfunction, which precedes plaque formation (22). However, it should be noted that the presence of significant correlations between the endothelial progenitor cell count, asymmetric dimethyl-L-arginine levels, the ratio of asymmetric dimethyl-L-arginine to L-arginine, and indices of atherogenesis does not imply a simple cause-and-effect relationship. Hill et al (21) have suggested that a depressed endothelial progenitor cell count might only reflect accelerated atherogenesis, since endothelial progenitor cell depletion can result from excessive endothelial progenitor cell utilization in the

ongoing repair of the continuously damaged endothelial layer. This sequence of events might aggravate blood endothelial progenitor cell deficiency, acting along with enhanced endothelial progenitor cell trafficking into the inflamed joints, with consequently lower endothelial progenitor cell counts in the presence of more advanced endothelial dysfunction and early atherogenesis. Accordingly, this can explain the inverse endothelial progenitor cell–IMT relationship found in the present study. Moreover, this concept is consistent with the observed correlations between the endothelial progenitor cell count and asymmetric dimethyl-L-arginine and between IMT and the ratio of asymmetric dimethyl-L-arginine to L-arginine, assuming that the source of asymmetric dimethyl-L-arginine would be the augmented breakdown of proteins originating from continuously injured endothelial cells undergoing increased apoptosis and turnover.

We cannot exclude the possibility that factors other than asymmetric dimethyl-L-arginine accumulation might simultaneously accelerate atherogenesis and reduce the endothelial progenitor cell count via an effect on endothelial progenitor cell mobilization and/or survival. In RA patients without classic risk factors, the capability of CRP to impair endothelial progenitor cell differentiation and survival at concentrations ≥15 mg/liter (56) and to produce direct proinflammatory effects on the endothelium at concentrations ≥5 mg/liter (58) suggests CRP as a possible candidate molecule. However, we did not observe a correlation between CRP levels and endothelial progenitor cell counts, which would have been expected if endothelial progenitor cell depletion in RA had been secondary to CRP elevation. Moreover, other investigators have not observed any relationship between the endothelial progenitor cell count and the CRP level in patients with RA with high (14) or low (15) disease activity, or in patients with CAD (23).

We did not perform in vitro assays, such as the colony-forming unit assay (21) or the endothelial progenitor cell culture assay (20), which are superior to flow cytometry in determining endothelial progenitor cell counts. Nevertheless, similar results have been obtained using flow cytometry and both in vitro assays, in studies comparing endothelial progenitor cell counts in patients with CAD (20,23,24,29) and RA (14) with counts in control groups, or in studies investigating changes in endothelial progenitor cell counts in response to statin therapy (34) or exercise-induced myocardial ischemia (59).

It should be noted that in some previous studies, CD133 positivity (typical for less mature endothelial progenitor cells) was included in the criteria used to identify endothelial progenitor cells by flow cytometry (CD34+,CD133+ [29] or CD34+,KDR+,CD133+ [14]). However, comparable results were obtained with endothelial progenitor cells defined as CD34+,KDR+ cells and those defined as CD133+ cells, with regard to the risk of future cardiovascular events (24). Additionally, negative correlations of the endothelial progenitor cell count with risk factors and beneficial effects of statins were found with endothelial progenitor cells considered as CD34+,KDR+ cells but not with those defined as CD34+,CD133+ or CD133+ cells (20,34). Finally, in many previous studies (14,15,20,23,24,29,34), the endothelial progenitor cell count, when quantified by flow cytometric analysis, was expressed as a percentage of mononuclear cells in the lymphocyte gate, similar to the method used in the present study.

Given the limitations discussed above, treatment strategies combining antiinflammatory properties with the ability to increase the circulating endothelial progenitor cell count might be expected to attenuate RA-associated cardiovascular risk. Accordingly, further research on the use of statins in RA can be recommended, since these drugs, in addition to having immunomodulatory properties, are able to elevate the endothelial progenitor cell count by ~200% in patients with CAD during 3–4 weeks of therapy (34). Indeed, both the antiinflammatory action of statins (60) and their ability to correct endothelial dysfunction (61) have been demonstrated in patients with RA. Finally, counteracting the imbalance between the substrate and endogenous inhibitor of NO synthesis by long-term treatment with L-arginine supplements might also be a topic of further research in RA.

AUTHOR CONTRIBUTIONS

Dr. Surdacki had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Surdacki, Wieczorek-Surdacka, Bode-Böger.

Acquisition of data. Martens-Lobenhoffer, Wloch, Marewicz, Rakowski.

Analysis and interpretation of data. Surdacki, Martens-Lobenhoffer, Wloch, Marewicz, Rakowski, Dubiel, Pryjma.

Manuscript preparation. Surdacki, Wloch, Marewicz, Rakowski, Wieczorek-Surdacka, Dubiel, Pryjma, Bode-Böger.

Statistical analysis. Surdacki.

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