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Homocysteine and antiphospholipid antibodies in rheumatoid arthritis patients: Relationships with thrombotic events

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ABSTRACT

Objective

To investigate the possible relationships between plasma homocysteine levels and thrombotic events in a select population of rheumatoid arthritis (RA) patients with or without antiphospholipid (aPL) antibody positivity.

Methods

168 female RA patients attending the Extra-articular Involvement RA Clinic of University of Genova and 72 female subjects matched for age and vascular diseases as controls were included in the study. 30 of the RA patients showed aPL antibody positivity and 138 aPL antibody negativity on the basis of the concomitant presence or absence of high concentrations of anticardiolipin (aCL) antibodies or the presence of lupus anticoagulant (LA). All subjects were evaluated for plasma homocysteine concentrations and for the occurrence of thrombotic events.

Results

Twenty-five RA patients and 5 controls reported a history of thrombotic events. Eleven and 5 of RA patients were found to have been previously affected by venous or arterial thrombosis, respectively. Plasma levels of homocysteine in aPL antibody positive patients with thrombosis were found to be significantly higher than in aPL antibody positive RA patients without thrombosis ($p < 0.001$). When RA patients with thromboses were analyzed, a significant increase of plasma homocysteine levels was found in aPL antibody-positive RA patients versus aPL antibody-negative RA patients ($p < 0.04$) and versus related controls ($p < 0.003$).

Conclusions

The association observed between aPL antibody positivity and high levels of plasma homocysteine in RA patients may represent a possible risk factor for thrombotic events. Therefore, it is suggested that hyperhomocysteinemia might be involved in the vascular-related mortality observed in RA patients with a history of thrombosis.

Introduction

Antiphospholipid (aPL) antibodies represent a family of closely related immunoglobulins that react with anionic phospholipids. aPL antibodies are defined as lupus anticoagulant (LA) if their presence prolongs the phospholipid-dependent coagulation tests. aPL antibodies are also defined as anticardiolipin (aCL) antibodies if they react with cardiolipin in the enzyme-linked immunosorbent assay (ELISA). The presence of aPL antibodies has been primarily associated with an increased risk for both arterial and venous thrombosis and may occur in patients with acquired antiphospholipid syndrome, but also in patients with autoimmune diseases, including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (1). aPL antibody positivity correlates strongly with the concomitant appearance of thromboembolic complications, such as peripheral vessel diseases, and cerebral and myocardial infarction.

Previous reports published by us and others have shown increased concentrations of aCL antibodies in patients affected by RA, with an increased risk of different vascular complications, including arterial or venous thrombosis, and in general vasculitis (2,3). On the other hand, recent studies reporting the frequency of atherosclerotic complications among patients with homocystinuria have helped to establish that elevated plasma levels of total homocysteine may be considered as an independent risk factor for arterial as well as venous thrombosis (4). Both genetic (enzymopathies) and non-genetic factors (nutritional deficiency of vitaminic cofactors, chronic disease, drug therapy) may be involved in the development of hyperhomocysteinemia (5, 6).

The present study reports on homocysteine level evaluations in RA patients showing the presence of aPL antibodies, and with an history of arterial and/or venous thrombosis based on a retrospective cohort study.

Material and methods

Patients

One hundred and sixty-eight female patients fulfilling the 1987 American Rheumatism Association Criteria for adult RA were selected from our Extra-articular Involvement RA Clinic (EIRAC), a secondary referral center

for Genoa and the surrounding area (7). The patients were affected by frequent RA complications such as vasculitis, renal and cardiovascular diseases. The mean age of the patients was 50 ± 11 years (mean ± SD). To establish normal reference values, a group of 72 female subjects matched for age and vascular diseases was evaluated (mean age 52 ± 9 years).

None of the patients had been treated with corticosteroid or intra-articular steroid injections during the 3 months preceding the start of the study. The patients were not under treatment with methotrexate, azathioprine, cyclophosphamide, or hydroxycloroquine. RA patients and related controls were matched for other risk factors such as lipid profile and body mass index.

Regarding vascular involvement, episodes of cerebral infarction were diagnosed based on clinical findings confirmed by lesions noted on computer tomography (CT) or magnetic resonance imaging. The diagnosis of deep vein thrombosis was confirmed by doppler ultrasound examination. The diagnosis of myocardial infarction was based on resting and exercise electrocardiogram, the enzyme activity pattern, angiography and CT.

Five of 72 controls had a history of thrombotic events [deep vein thrombosis in the lower (4 cases) or upper (1 case) limbs]. The controls with thrombotic events had been affected by traumatic articular injuries.

Detection of aPL antibodies

Briefly, for the presence of LA the dilute prothrombin time and dilute activated partial thromboplastin time were measured and the concordance of both tests was considered. Positive samples were tested for the dilute Russell's Viper Venom Time (dRVVT: IL Test LAC Screen, Instrumentation Laboratory, Milan, Italy) and were retested after 1:1 mixing with normal plasma. If the clotting time of the mixed plasma sample still was ≥ 20% longer than normal plasma, the dRVVT was repeated with an addictional amount of phospholipids (IL Test LA Confirm, Instrumentation Laboratory, Milan,Italy). Samples with a ratio of the IL Test screen/LA confirm > 1.2 were considered positive for LA.

Serum levels of IgG-type aCL antibodies and IgM-type aCL were measured by ELISA on at least two occasions, as previously described (2). aCL antibodies (IgG and IgM) were measured in duplicate using a commercial anticardiolipin antibody kit (Diastat, Bouty Diagnostici, Milan, Italy). Briefly, serum samples were diluted 10 l in sample diluent (phosphate-buffered saline, pH 7.4, containing 10% bovine serum). 100 l of diluited serum were incubated in duplicate wells of 96-well plates coated with cardiolipin antigen. The plates were washed and anti-human alkaline phosphatase conjugated IgG and IgM was added to each well and incubated for 30 minutes at room temperature. The plates were washed 3 times and the reaction was blocked by the addition of a stop solution. The absorbance at 550 nm was measured. This assay was standardized relative to international reference preparations obtained from the Antiphospholipid Standardization Laboratory (University of Louisville, Kentucky, USA). Values are expressed in GPL and MPL Units. The mean values +5 standard derivation of 86 healthy controls (15 GPL Units and 10 MPL Units) evaluated in our clinic were considered as cut-off for the IgG and IgM aCL levels, respectively.

Determination of plasma homocysteine levels

Total plasma homocysteine levels were determinated by high performance liquid chromatography according to Fortin *at al*. (8). Briefly, plasma samples were reduced by tri-n-butylphosphine, deproteinated by perchloric acid and then derivatised using the thiol-specific reagent ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate. Chromatographic separation was performed isocratically at flow rate of 0.6 ml/min on an octadecyl column. The mobile phase was 2% methanol in 10 mM acetate buffer (pH4.0). The derived thiols were detected by fluorescence at an excitation wavelength of 380 nm, emission 515 nm.

Results

Table I shows the clinical characteristics of the patients and their antiphos-

Table I. Characteristic and frequency of the lupus anticoagulant (LA) and anticardiolipin antibodies (aCL) in RA patients with and without thromboses. All values are expressed as the mean ± SD. IgM-RF = IgM Rheumatoid Factor.

	No thromboses (n = 148)	Arterial thromboses (n = 9)	Venous thromboses (n = 11)
Age (years ± SD)	50±9	49 ± 2	50 ± 3
Disease duration (years ± SD)	9±4	8 ± 2	10 ± 3
ESR (mm/hour ± SD)	34±16	39 ± 14	38 ± 12
IgM-RF (positive)	130 (88%)	8 (89%)	10 (91%)
LA (-) / aCL (+)	8 (5%)	5 (56%)	7 (64%)
aCL Isotypes:			
IgG (GPL Units ± SD)	n=3; 42 ± 9	n=4; 53 ± 11	n=3; 55 ± 10
IgM (MPL Units ± SD)	n=2; 52 ± 8	n=1; 48	n=2; 48 ± 9
IgG and IgM	n=3		n=2
GPL Units	46 ± 7		50 ± 9
MPl Units	39 ± 8		41 ± 6
LA (+) / aCL (+)	1 (0.6%)	3 (33%)	4 (36%)
aCL Isotypes:			
IgG (GPL Units)	n=1; 54	n=2 ; 64 ± 5	n=2 ; 48 ± 10
IgM (MPL Units)		n=1 ; 43	n=1 ; 46
IgG and IgM			n=1
GPL Units			53
MPl Units			48
LA (+) / aCL (-)	1 (0.6%)	1 (11%)	0

pholipid status, according to the presence of LA and/or aCL antibodies, and divided on basis of a history with and without thrombosis.

The total group of RA patients was found to be affected by a significantly higher rate of venous and/or arterial thrombosis when compared to the controls [n = 25/168 (12%) vs 5/72 (6%) respectively, $p < 0.01$]. Among aPL antibody-positive RA patients 11 (7%) had venous thrombosis (most frequently deep venous thrombosis of the legs), and 9 (5%) had arterial thrombosis (involving the coronary and cerebral vessels in most cases). Among the aPL negative RA patients 5 were affected by venous thrombosis (only deep venous thrombosis of the legs).

The presence of LA (+) / aCL (+) was found in 7/20 patients with thrombosis (35%) and in one (0.6%) patient without thrombosis. Samples with LA (-) / aCL (+) were observed in 12 (60%) and 8 (5%) of the patients with and without thrombosis, respectively. Samples with LA (+) / aCL (-) were found only in one (11%) patient with arterial thrombosis and in one (0.6%) patient without thrombosis (see Table I).

Interestingly, plasma total homocysteine levels were found to be significantly increased in all patients affected by RA when compared to related controls (14.5± 5.4 mol/l vs 9.3 ± 4.1 mol/l; $p < 0.001$) as well as in aPL antibody-positive RA patients when compared in aPL antibody-negative RA patients and matched controls (16.6 ± 5.6 mol/l vs 13.9± 5.1 mol/l and 9.3± 4.1 mol/l, respectively; $p < 0.01$ and $p < 0.0001$). When we analyzed the patients affected by thromboses, significantly higher levels of homocysteine were found in aPL antibody-positive RA patients versus aPL antibody-negative RA patients (22 ± 4.1 mol/l vs 17.4 ± 2.4 mol/l; $p < 0.04$) as well as versus matched controls (13.6 ± 2.6 mol/l; $p < 0.003$) (Fig. 1).

Therefore, plasma levels of homocysteine in aPL antibody-positive patients with thromboses, were significantly higher than in aPL antibody-positive RA patients without thromboses (22 ± 4.1 mol/l vs 12.8 ± 2.5 mol/l, $p < 0.001$), as well as in aPL antibody-negative RA patients with versus without thromboses (17.4 ± 2.4 mol/l vs 13.8 ± 5.1 mol/l; $p < 0.02$) and matched controls (13.6 ± 2.6 mol/l vs 9.1 ± 4.1 mol/l; $p < 0.02$) (Fig. 1). Interestingly, homocysteine levels in aPL antibody-positive RA patients with arterial thromboses were found higher than in those with venous thromboses, however, the difference was not statistically significant (22.8± 4.2 mol/l vs 20.7 ± 3.1 mol/l, $p < 0.07$).

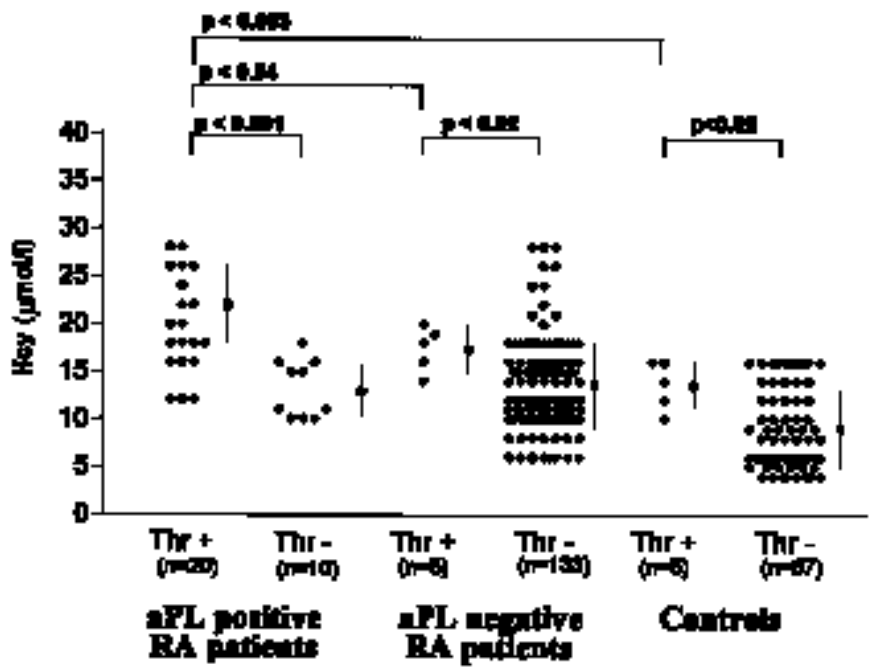

Fig. 1. Plasma levels of homocysteine (Hcy) in antiphospholipid antibody-positive, antiphospholipid antibody-negative rheumatoid arthritis (RA) patients and related controls. Thr+ = patients with thrombosis; Thr- = patients without thrombosis.

Discussion

These findings suggest that elevated plasma levels of homocysteine may be associated with a past history of thrombotic events in patients affected by both RA and aPL antibody-positivity. In the last two decades, a growing amount of attention has been focused on hyperhomocysteinemia as a risk factor for thromboembolic diseases (9).

Higher homocysteine concentrations are well known to occur in patients with other pathologic conditions such as renal failure, cobalamin and folate deficiencies, as well as hypothyroidism and inflammatory bowel disease, suggesting a potential mechanism for the high incidence of thrombotic complications in these patients (10, 11). The observed increased homocysteine concentrations in RA patients might be a consequence of several nutritional abnormalities, including low circulating vitamin B 6 levels due to the reduction in circulating levels of pyridoxal 5'-phosphate, the biologically active form of vitamin B6 (12). The mechanism(s) by which hyperhomocysteinemia might contribute to atherogenesis and thrombogenesis are largely understood.

The individual risk of thrombosis in a patients with congenital or acquired defect predisposing to thrombosis may vary considerably and other clotting abnormalities (i.e., factor V or prothrombin mutations) have been shown to be additional risk factors in both primary and secondary antiphospholipid syndrome (13,14). There is evidence that the presence of severe risk factors in the same subject potentiates the risk of thrombosis.

In conclusion, the reported association between aPL antibody positivity and high levels of plasma homocysteine in RA patients, may represent a consistent risk factor for thrombotic events, as already observed in SLE patients (15). Therefore, it is suggested that hyperho-

mocysteinemia might be involved in vascular-related mortality observed in RA patients with a history of thrombosis (16).

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