Antioxidant Vitamins and Lipid Peroxidation in Patients with Rheumatoid Arthritis: Association with Inflammatory Markers

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ABSTRACT. Objective. We evaluated vitamin status in relation to inflammatory markers and lipid peroxidation measures in patients with rheumatoid arthritis (RA).

Methods. Thirty patients with RA and 30 controls were studied. Lipid profile, vitamin A, vitamin E, and inflammatory markers were analyzed in all subjects. Susceptibility to low density lipoprotein (LDL) oxidation was evaluated in both groups by measuring the kinetics of conjugated dienes induced by hemin.

Results. Patients and controls had similar lipid profiles, except with LDL cholesterol, which was lower in patients (p < 0.05). Patients had significantly higher plasma levels of inflammatory markers with respect to controls (p < 0.01). Plasma levels of vitamin A were lower in patients, and similar levels of vitamin E were observed in both groups. Oxidative variables, measured as the different phases of conjugated diene formation, were similar in patients and controls. We found a significant inverse correlation between vitamin A, vitamin E, and secretory type II phospholipase A2 in patients. We found a positive correlation between the affinity constant of LDL binding to glycosaminoglycans (GAG), Kd-LDL, and the lag phase of LDL oxidation (p < 0.05) in patients.

Conclusion. This report supports the hypothesis that chronic inflammation affects antioxidant vitamin levels in RA. Combined with the presence of a chronic inflammatory process and high LDL affinity for GAG, this may explain the high risk of cardiovascular disease in patients with RA.

Rheumatoid arthritis (RA) is a chronic inflammatory disease that has been associated with an increase in cardiovascular diseases. The cause of increased risk for cardiovascular disease in patients with RA is unknown. Patients with active RA have dyslipoproteinemia characterized by decreased serum concentrations of total cholesterol and cholesterol in low density lipoproteins (LDL) and high density lipoproteins (HDL). Although this lipid profile in patients does not imply a higher atherogenic risk, some qualitative lipoprotein modifications, such as oxidative susceptibility, have not been evaluated. It has been postulated that the chronic inflammation associated with RA could lead to an accelerated atherogenesis. Among the factors that could contribute to this accelerated atherogenesis, lipid peroxidation could play an important role. Lipid peroxidation may contribute to initiation and progression of the atherosclerotic plaque. Oxidized LDL and the products released during oxidation of lipids are reported to induce a series of biological effects with consequences for atherogenesis. Oxidized LDL and its products are reported to modulate inflammatory response. High concentrations of lipid peroxides in synovial fluid and serum of patients with inflammatory joint diseases are reported. Further, Selley, et al found that the concentration of 4-hydroxynonenal, an aldehydic product derived from lipid peroxidation, is increased in patients with RA compared to patients with osteoarthritis.

Vitamin E and carotenoids protect LDL from oxidation, leading to an antiatherogenic effect due to their antioxidant capacity. Low antioxidant levels are associated with an increase in cardiovascular disease and RA. Some studies have described lower serum α-tocopherol and vitamin A
concentrations in patients with RA\textsuperscript{19}, although this was not confirmed by others\textsuperscript{20}. In addition, it has been suggested that vitamins E and A possess antiinflammatory activity\textsuperscript{21,22}. Vitamin E may directly affect the arachidonic acid cascade. Sakamoto, et al demonstrated that macrophages from vitamin E deficient rats secrete higher levels of prostaglandin (PG) E\textsubscript{2} that is related to the activation of phospholipase A\textsubscript{2} (PLA\textsubscript{2})\textsuperscript{23}. The activity of this enzyme was inhibited considerably by the administration of vitamin E\textsuperscript{24}. However, there are no data showing a relationship between vitamin status and PLA\textsubscript{2} in patients with RA. Further, inflammatory markers such as secretory type II PLA\textsubscript{2} (sPLA\textsubscript{2}-IIA), adhesion molecules (vascular cell adhesion molecule, VCAM; intercellular adhesion molecule, ICAM), and cytokines (tumor necrosis factor-\(\alpha\), TNF-\(\alpha\); interferon-\(\gamma\), IFN-\(\gamma\)) have been related to the inflammatory response and the pathogenesis of RA\textsuperscript{25-28} and also atherosclerosis\textsuperscript{29-33}. We evaluated vitamin status and lipid peroxidation indicators in relation to sPLA\textsubscript{2}-IIA, inflammatory markers, and proatherogenic properties of LDL in patients with RA.

MATERIALS AND METHODS

Study population. We studied 30 patients (27 women, 3 men) and 30 controls. Each patient was diagnosed as having RA according to the 1987 criteria of the American College of Rheumatology\textsuperscript{34}. All subjects were attending the Hospital del Mar, Barcelona, Spain. The mean age of patients at the time of study was 53 years (range 24–70) and the mean disease duration was 10 years (range 1–30). No patient had laboratory or clinical signs of kidney, liver, thyroid, or infectious disease, diabetes mellitus, or malignancy. Patients had not had previous stroke or myocardial infarction. Thirty age and sex matched healthy controls (27 women, 3 men) with no clinical or laboratory evidence of disease were recruited. The mean age of controls was 53.2 years (range 28–70). Patients and controls lived in the same area, shared similar eating patterns with no special dietary advice, and there was no difference in body mass index (BMI) between patients and controls (Table 1). The study was approved by the local committee for ethical clinical investigation.

Sample collection and lipid profile. Blood samples were taken after an overnight fast (12 h) and collected into tubes containing EDTA (1 mg/ml). After centrifugation (10 min at 2000 rpm at 4°C), 4 ml plasma samples were collected into polypropylene vials (Greiner BV, Alphen a/d Rijn, The Netherlands) to which 10 µM of butylated hydroxytoluene and 6 mg/ml of saccharose were added to prevent LDL oxidation and aggregation. The samples were stored at ~80°C until use.

Total plasma cholesterol and triglycerides were determined enzymatically (Roche Diagnostics Scandinavian AB, Bromma, Sweden). Plasma apolipoprotein B (apoB) and apolipoprotein A-1 (apoA-1) concentrations were measured using an immune nephelometric method. Lipoprotein(a) [Lp(a)] concentrations (mg/dl) were determined by an immunological method (SPQ test system; DiaSorin) adapted in a Cobas Mira autoanalyzer. Vitamin E and A concentrations in plasma. Concentrations of vitamins A and E were determined by high pressure liquid chromatography (Hewlett Packard 1050) equipped with a UV-visible detector, using retinol acetate and tocopherol acetate as internal standards. The column was a Spherisorb ODS (125 \times 4 mm, 5µ) and the mobile phase was methanol:water (100% for vitamin E and 98% for vitamin A)\textsuperscript{35}.

Hemin oxidation of LDL. LDL was isolated from plasma by sequential preparative ultracentrifugation\textsuperscript{36} in a Kontron 45.6 rotor (Teknicron T1055, Kontron Instruments, Sweden). Before the start of the oxidation of LDL, samples were desalted and EDTA removed by filtration PD10 columns containing Sephadex G-25 M (Amersham Pharmacia). The samples were balanced by phosphate buffered saline (10 nM sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride). To study the susceptibility of LDL to oxidation, the kinetics of conjugated diene formation induced by hemin was used\textsuperscript{37}. Hemin, a product of \emph{in vivo} hemoglobin degradation, binds and oxidizes lipoproteins. The kinetics of LDL oxidation (0.1 mg apoB/ml) were determined by monitoring the change in absorbance at 250 nm at 30°C with a UV spectrophotometer (Uvikon 922, Kontron) in the presence of hemin (2.5 µM) and H\textsubscript{2}O\textsubscript{2} (10 µM) for 4 h. Hemin oxidation of LDL was divided into 3 characteristic indices. The lag phase is defined as the time interval (min) in which endogenous antioxidants (i.e., \(\alpha\)-tocopherol) are consumed. The maximal rate (mol dienes/mol LDL \times min) is an index derived from the propagation phase that begins with acceleration of oxidation of the polynsaturated fatty acids. The maximum diene production (mol dienes/mol LDL) represents the maximal amount of dienes produced during LDL oxidation and depends on the content of peroxidation substrates. The 3 variables (lag phase, maximal rate, maximum diene production) give more information on the susceptibility of LDL oxidation and their use has been widespread.

Analysis of plasma inflammatory markers and other variables. Plasma levels of sPLA\textsubscript{2}-IIA were measured using an ELISA system with a monoclonal antibody (Cayman Chemical Co., Ann Arbor, MI, USA) for capture and polyclonal antibody with colorimetric detection. Soluble adhesion molecule (VCAM-1, ICAM-1), TNF-\(\alpha\), and IFN-\(\gamma\) plasma concentrations were measured with commercial ELISA kits (R&D Systems Europe, Ltd.). C-reactive protein (CRP) was measured using a commercial kit (Medix Biochemica, Finland). Other variables such as fibrinogen, rheumatoid factor (RF), hemoglobin (Hb), erythrocyte sedimentation rate (ESR), and hepatic and renal function were measured using standard clinical methods. LDL oxidation and interaction with matrix glycosaminoglycans. Interaction between LDL and chondroitin-6-sulfate (C6S) glycosaminoglycan (GAG) (Seikagaku Corp., Tokyo, Japan) at physiological salt concentrations was measured by electrophoretic mobility shift assay\textsuperscript{38}. Results are expressed as affinity constant of LDL binding (Kd-LDL), i.e., lower Kd equals higher affinity of LDL to GAG.

Statistics. Results are presented as means \pm standard deviation. The significance of difference between patient and control groups was analyzed by

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Table 1. Morphometric and clinical characteristics of control subjects and patients with RA.

<table>
<thead>
<tr>
<th></th>
<th>Controls, n = 30</th>
<th>Patients, n = 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>53.2 ± 12.6</td>
<td>53.0 ± 13.1</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body mass index, kg/m\textsuperscript{2}</td>
<td>27.4 ± 4.4</td>
<td>26.0 ± 5.2</td>
</tr>
<tr>
<td>Disease duration, yrs</td>
<td>10 ± 7.9</td>
<td>9 ± 7.9</td>
</tr>
<tr>
<td>Rheumatoid factor, % positive</td>
<td></td>
<td>93.3</td>
</tr>
<tr>
<td>Rheumatoid factor, value</td>
<td>242.0 ± 56.4</td>
<td></td>
</tr>
<tr>
<td>HAQ, range 0–3</td>
<td>1.7 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Swollen joint count, range 0–66</td>
<td>13.2 ± 5.6</td>
<td>13.2 ± 5.6</td>
</tr>
<tr>
<td>Tender joint count, range 0–68</td>
<td>18.4 ± 8.0</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients taking DMARD, %</td>
<td>86.6</td>
<td></td>
</tr>
<tr>
<td>Patients taking NSAID, %</td>
<td>93.3</td>
<td></td>
</tr>
<tr>
<td>Patients taking corticoids, %</td>
<td>90.0</td>
<td></td>
</tr>
<tr>
<td>Doses of prednisone equivalent, mg/day</td>
<td>6.5 ± 2.8</td>
<td></td>
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</tbody>
</table>

Except where otherwise indicated, values are mean ± SD. HAQ: Health Assessment Questionnaire; DMARD: disease modifying antirheumatic drugs; NSAID: nonsteroidal antiinflammatory drugs.
RESULTS
Characteristics of subjects. Table 1 shows that controls and patients with RA were of similar age, sex, and BMI without significant differences. The majority of patients with RA were positive for RF (93.3%) and had erosive arthritis (90%). Patients were receiving treatment with nonsteroidal antiinflammatory drugs (NSAID) (93.3%), corticosteroids (90%), and disease modifying antirheumatic drugs (DMARD) (86.6%).

As expected, CRP and ESR levels were significantly higher in patients compared to controls (p < 0.001) (Table 2). Plasma concentrations of other inflammatory markers, sPLA2-IIA, fibrinogen, ICAM, IFN-γ, and TNF-α, were also significantly higher in patients (p < 0.001 for sPLA2-IIA, fibrinogen, ICAM, TNF-α; and p < 0.01 for IFN-γ) (Table 2). The results showed a positive and significant correlation between sPLA2-IIA and CRP (r² = 0.743, p < 0.001) (Table 2).

Table 2. Inflammatory marker levels in plasma of patients with RA and control subjects.

<table>
<thead>
<tr>
<th></th>
<th>Controls, n = 30</th>
<th>Patients, n = 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP, ng/l</td>
<td>0.16 ± 0.2</td>
<td>3.7 ± 2.1**</td>
</tr>
<tr>
<td>ESR, mm/h</td>
<td>14.9 ± 10.5</td>
<td>67.7 ± 25.6**</td>
</tr>
<tr>
<td>Fibrinogen mg/dl</td>
<td>371.3 ± 72</td>
<td>525.0 ± 95.7**</td>
</tr>
<tr>
<td>sPLA2-IIA, ng/ml</td>
<td>14.0 ± 8.6</td>
<td>96.2 ± 76.3**</td>
</tr>
<tr>
<td>ICAM, ng/ml</td>
<td>248.1 ± 74.6</td>
<td>344 ± 130.6**</td>
</tr>
<tr>
<td>VCAM, ng/ml</td>
<td>479.8 ± 105.5</td>
<td>559.6 ± 227.6</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>11.6 ± 2.8</td>
<td>20.6 ± 17.4**</td>
</tr>
<tr>
<td>IFN-γ, pg/ml</td>
<td>1.9 ± 2.8</td>
<td>5.4 ± 5.8*</td>
</tr>
</tbody>
</table>

Values are mean ± SD. *p < 0.01; ** p < 0.001 versus controls.


Table 3. Lipid and lipoprotein levels in plasma of patients with RA and control subjects.

<table>
<thead>
<tr>
<th></th>
<th>Controls, n = 30</th>
<th>Patients, n = 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma cholesterol, mg/d</td>
<td>213 ± 41.4</td>
<td>200 ± 44.0</td>
</tr>
<tr>
<td>Plasma triglycerides, mg/dl</td>
<td>107 ± 41.0</td>
<td>105 ± 63.1</td>
</tr>
<tr>
<td>Plasma apoB, g/l</td>
<td>1.16 ± 0.3</td>
<td>1.08 ± 0.3</td>
</tr>
<tr>
<td>Plasma apoA-1, g/l</td>
<td>1.41 ± 0.2</td>
<td>1.47 ± 0.2</td>
</tr>
<tr>
<td>Lp (a), mg/dl</td>
<td>21 ± 18.0</td>
<td>40 ± 41.0</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>148 ± 41.2</td>
<td>134 ± 35.6*</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>51 ± 12.4</td>
<td>53 ± 14.6</td>
</tr>
</tbody>
</table>

Values are mean ± SD. *p < 0.05 versus controls.

LDL: low density lipoprotein; apo: apolipoprotein, HDL: high density lipoprotein.

DISCUSSION
This is the first study to evaluate vitamin status in relation to inflammatory markers and proatherogenic properties of LDL in patients with RA. We found a significant inverse correlation between vitamin A, vitamin E, and sPLA2-IIA and between these vitamins and CRP in patients with RA. These results support the hypothesis that chronic inflammation affects antioxidant vitamin levels in RA.

Studies indicate that free radicals in chronic inflammatory conditions associated to RA and atherosclerosis may contribute to the pathogenesis of these diseases. There is evidence that inflammatory cells such as neutrophils,
lymphocytes, and macrophages are present in synovial fluid and atherosclerotic plaque. The activity of these cells, particularly macrophages, produces compounds including free radicals that can initiate the free radical-driven lipid peroxidation process. This reaction happens in polyunsaturated fatty acids contained in the cell membrane phospholipids and can produce cell damage. The increase in oxidative stress due to inflammatory cells may explain why antioxidant vitamins decrease at the same time that inflammatory markers increase in patients with RA.

In patients with RA, we found lower levels of vitamin A and similar levels of vitamin E compared with matched controls. No differences were observed in LDL diene formation induced by hemin between the 2 groups. Although these results were unexpected, it is important to note that patients with RA were under treatment with NSAID and DMARD.

Figure 1. Concentrations of vitamin A and vitamin E and oxidative variables in controls and patients. Oxidation variables are derived from the different phases of conjugated diene formation induced by hemin: lag phase, maximal rate, and maximum diene production. Results expressed as mean ± SD.
From these data we cannot attribute the increased susceptibility to myocardial infarction in patients with RA to higher LDL oxidation in plasma.

Treatment in patients with RA can modify their lipid profile and antioxidant status. Administration of indomethacin inhibits lipid peroxidation in rat adjuvant arthritis. Moreover, studies in patients with active RA have revealed some alterations in lipid profile characterized by a decrease in plasma lipoproteins (very low density lipoprotein, LDL, HDL). These alterations were normalized with a treatment that regulates the activity of the disease. Our study showed that despite treatment patients with active RA had lower LDL cholesterol than controls. No significant differences were observed in other lipid profile variables we studied. Lp(a) is associated positively with cardiovascular disease. Plasma levels of Lp(a) are reported to be elevated in RA.

Figure 2. Relationship of vitamin A and vitamin E to inflammatory markers — CRP, secretory type II PLA₂ (sPLA₂-IIA), and fibrinogen — in patients with RA.
Our study showed that plasma Lp(a) levels were higher in patients with RA than in controls, but did not reach statistical significance. One explanation for this may be the small number of patients and controls used in this study. Patients with RA, even undergoing antiinflammatory treatment, showed disturbances in their antioxidant status and in the characteristics of their LDL, such as increased affinity for GAG and susceptibility for hemin oxidation, that put them at higher risk of cardiovascular heart disease.

We have reported that plasma from patients with RA had significantly higher concentrations of small, dense LDL with high affinity for GAG compared to control subjects. Small and dense LDL particles are more atherogenic due to their high affinity for proteoglycans in the arterial intima. These LDL are taken up by macrophages, leading to the formation of foam cells. Moreover, the retention of LDL and the lag phase of diene formation in patients with RA were significantly longer than in controls, but did not reach statistical significance. One explanation for this may be the small number of patients and controls used in this study.

Atherogenesis, as RA, is a chronic inflammatory process in which lipid peroxidation and antioxidant vitamins play an important role. Moreover, inflammatory markers such as sPLA₂-IIA and CRP that are increased in patients with RA have been associated to high risk of cardiovascular disease. The disturbances in the antioxidant status and high LDL affinity for GAG combined with the presence of chronic inflammation may contribute to an explanation of the high risk of cardiovascular disease in patients with RA.

ACKNOWLEDGMENT
We are grateful to the clinical laboratory and the patients from the Hospital del Mar for making this study possible. We thank the Unitat de Recerca de Lípids i Arteriosclerose and the Wallenberg Laboratory, Göteborg, Sweden, for technical assistance.

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