Decrease of Serum Ascorbic Acid Concentrations in Patients with Diabetic Macroangiopathy

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Summary The relationship between serum ascorbic acid (AA) and diabetic macroangiopathy was studied. Fifty-six patients with non-insulin-dependent diabetes mellitus were examined, together with 20 healthy controls matched for age against the diabetes patients. Aortic pulse wave velocity (PWV) was taken as an index of the severity of atherosclerosis. The level of serum AA in diabetic patients was significantly lower than that of the controls. Among the diabetic groups, those with elevated PWV levels by age demonstrated a significant drop in AA. No significant differences were seen in the level of serum dehydroascrobic acid (DHAA) between patients and controls, nor were there any significant differences among patient groups. The concentration of serum AA was inversely related to the risk factors of atherosclerosis, such as body mass index, Apo B/Apo A-I ratio, thiobarbituric acid-reactive substances (TBARS), and microalbumin in urine. It was inferred from these findings that the depletion of serum AA was apparent in diabetics with advanced atherosclerosis.

Key Words ascorbic acid, lipid peroxide, diabetic macroangiopathy, aortic pulse wave velocity

Plasma lipid peroxide (LPO) has been reported to play an important role in the onset and development of atherosclerosis in patients with coronary (1-3) or peripheral artery disease (1, 3, 4). LPO levels are also high in patients with diabetes mellitus, and free radicals have been implicated in the development of atherosclerosis (5). LPO generation is known to depend upon the action of free radicals. Recent studies have shown that free radicals are generated from glycosylated

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proteins in the presence of a high blood glucose concentration (6–8), and the
glycation of Cu- and Zn-superoxide dismutase (SOD) lowers SOD activity (9).

Organisms defend against the free radical-LPO system with enzymes, proteins,
and vitamins. Of all these compounds, ascorbic acid (AA) is a powerful antioxi-
dant in aqueous solution (10) and in human plasma (11). Fat-soluble vitamin E is
a powerful antioxidant in the cell membrane or lipoprotein, and it is known that the
byproduct generated by the antioxidative reaction, a vitamin E radical, is reduced
by aqueous AA to reconstruct vitamin E (12–14). The same kind of reaction is
observed with human low-density lipoprotein (LDL) (15). Furthermore, the
synthesis of collagen and proteoglycans decreases when the intake of AA in cells is
restricted in the fibroblast culture system with high blood glucose concentrations
(16). This finding suggests that cytotoxically damaged vascular endothelium can
be caused by an abnormality in collagen synthesis under the conditions of AA
deficiency. The present study examined the effect of serum AA and dehydroascor-
bic acid (DHAA) on diabetic macroangiopathy.

PATIENTS AND METHODS

Patients. Fifty-six patients (28 men and 28 women), ranging in age from 40
to 65 years, with non-insulin-dependent diabetes mellitus (NIDDM) were selected
as subjects for this study. Patients with nephrosis or liver disease were excluded.
Twenty normal subjects (6 men and 14 women), ranging in age from 40 to 65 years,
were chosen to form a control group whose medical status was checked by
thorough clinical examinations. The patients were classified as hypertensive if they
were currently taking antihypertensive medication, if their systolic pressure was
≥160 mmHg or if their diastolic pressure was ≥95 mmHg. Retinopathy was
assessed by fundus photography after pupillary dilation and classified as normal,
background retinopathy, or proliferative retinopathy. In the study group, 25 were
normal, 23 had background retinopathy, and 8 had proliferative retinopathy.

Aortic pulse wave velocity. Aortic pulse wave velocity (PWV) was measured
with a PWV-200 (Fukuda Denshi) as an index of the severity of atherosclerosis.
PWV is expressed as meters per second, measuring the pulse wave velocity from the
aortic ostium to the pulse portion of femoral artery. In proportion to the
development of atherosclerosis, the blood vessel is hard or the arterial lumen is
narrow, and PWV becomes faster. Hasegawa (17) measured PWV in 106,559
healthy people, expressed as M ± SD every one year of age. Their data has been
generally used as the standard for the assessment of atherosclerosis in Japan. The
NIDDM patients were divided into four groups applying this standard: Group A
(≤M + 1SD), Group B (>M + 1SD, ≤M + 2SD), Group C (>M + 2SD, ≤M +
3SD) and Group D (>M + 3SD).

In order to eliminate the effects of age, the difference in PWV levels between
the average PWV score of the controls and the PWV score of a NIDDM patient of
the same age was determined to be ΔPWV.
Measurement of serum AA and DHAA. A blood sample was collected from each patient in the morning before breakfast, and 5 ml of the sample was immediately shielded from light and serum was separated and stored at \(-80^\circ\text{C}\). The amount of AA and DHAA were measured within one week. A modified Okamura’s method (18) with dithiothreitol (DTT) was used to measure the amount of DHAA. The total AA value was obtained by adding 0.1 ml of a 0.1% DTT solution to 1.0 ml of serum. After a 30-min incubation period at 25\(^\circ\text{C}\), 1.0 ml of 10\% metaphosphoric acid was added and the mixture was placed in an ice bath for 10 min. Finally, the mixture was centrifuged at 3,500 \(\times g\) for 3 min at 4\(^\circ\text{C}\), and the supernatant was collected to measure the amount of AA. The DHAA value is expressed as the difference between the amount of AA measured in serum without DTT and the total AA value. The AA value was measured by high-performance liquid chromatography (HPLC) at a wavelength of 268 nm on a LiChrosorb NH\(_2\) column, using acetonitril mixed with 0.01 M KH\(_2\)PO\(_4\) (75:25) as the eluent. L-Ascrobic acid, manufactured by Wako Pure Chemical, Tokyo, was used as the standard solution of AA, and its purity was tested by titration using a 0.001 N potassium iodate solution.

Additional biochemical tests. Serum samples taken from fasting patients were used to measure the level of fasting blood glucose (FBG) by the glucose-oxidase method; glycosylated HbA\(_{1c}\) (HbA\(_{1c}\)) by the HPLC method; total cholesterol (TC) and triglyceride (TG) using a Hitachi 736 autoanalyzer; HDL-cholesterol (HDL-C) by the precipitation method with magnesium chloride; apolipoprotein by the immunoturbidimetry method (Daiichi Pure Chemicals kit) and serum LPO by the method of Yagi (19) in which serum LPO is expressed as a thiobarbituric acid-reactive substance (TBARS). The amount of microalbumin in urine was measured by the latex method, and is expressed in the form of mg/g of creatinine.

Statistical analysis. Data are expressed as M\(\pm SD\). The chi-square test was used to compare the frequency of hypertension, retinopathy and proteinuria in groups A, B, C and D. A multiple comparison test by Scheffe’s F was used to assess other clinical conditions. Pearson’s correlation coefficient was used to examine the relationship between the value of total AA, AA, and DHAA, and the risk factors of atherosclerosis.

RESULTS

When compared to grups A, B, and C, the frequency of hypertension in group D was higher. The frequency of retinopathy in groups C and D was significantly higher than that in group A (Table 1).

The concentration of FBG in group D was higher than that in group A, and the HbA\(_{1c}\) level in group D was higher than that in group A (Table 2). There were no marked differences in the amount of TC, TG, HDL-C, or the atherogenic index among diabetic groups. The serum TBARS levels in group D was significantly higher than that in normal subjects.
Table 1. Clinical features of the diabetic patients classified by PWV levels.

<table>
<thead>
<tr>
<th></th>
<th>Normal subjects</th>
<th>Patients with NIDDM (PWV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Group A</td>
</tr>
<tr>
<td>$N$</td>
<td>20</td>
<td>32</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>52±7</td>
<td>54±7</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>6/14</td>
<td>16/16</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.6±2.3</td>
<td>22.7±2.7</td>
</tr>
<tr>
<td>Duration of DM (yrs)</td>
<td>—</td>
<td>11±6</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>0</td>
<td>34.3</td>
</tr>
<tr>
<td>Retinopathy (%)</td>
<td>0</td>
<td>37.5</td>
</tr>
<tr>
<td>Albuminuria (%)</td>
<td>0</td>
<td>31.2</td>
</tr>
<tr>
<td>Microalbuminuria</td>
<td></td>
<td>12.5</td>
</tr>
<tr>
<td>Macroalbuminuria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>Diet</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Oral agent</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>8</td>
</tr>
</tbody>
</table>

PWV, aortic pulse wave velocity; Diabetic subjects were classified as follows using the standard deviation from PWV levels by age, as described by Hasegawa et al. Group A: $\leq M \pm 1SD$; Group B: $> M + 1SD, \leq M + 2SD$; Group C: $> M + 2SD, \leq M + 3SD$; Group D: $> M + 3SD$.

* $p<0.05$, ** $p<0.01$ vs. group A.

Table 2. Laboratory data of the diabetic patients classified by PWV levels.

<table>
<thead>
<tr>
<th></th>
<th>Normal subjects</th>
<th>Patients with NIDDM (PWV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Group A</td>
</tr>
<tr>
<td>FBG (mg/dl)</td>
<td>88±10</td>
<td>149±59**</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>—</td>
<td>8.1±1.8</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>195±26</td>
<td>219±36</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>70±30</td>
<td>134±115</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>53±12</td>
<td>53±12</td>
</tr>
<tr>
<td>AI</td>
<td>2.7±0.7</td>
<td>3.2±1.2</td>
</tr>
<tr>
<td>TBARS (nmol/ml)</td>
<td>4.3±1.1</td>
<td>5.4±1.8</td>
</tr>
</tbody>
</table>

FBG, fasting blood glucose; HbA₁c, (glycosylated) hemoglobin A₁c; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; AI, atherogenic index; TC−HDL-C/HDL-C; TBARS, thiobarbituric acid-reactive substances.

* $p<0.05$, ** $p<0.01$ vs. normal subjects.

The serum AA concentration in the diabetic patients was lower (705±257 μg/dl) than that of the controls (898±214 μg/dl) ($p<0.01$). There was no significant difference in the serum AA concentration among the controls, group A (805±245 μg/dl) and group B (645±276 μg/dl). However, the serum AA concentration was significantly lower in group C (525±213 μg/dl) and not significantly lower in group D.
low in group D (586±150 μg/dl) compared with the control (respectively \( p = 0.0019, p = 0.0574 \)) (Fig. 1). Serum AA was significantly low in group C compared with that in Group A (\( p = 0.0207 \)). There were no significant differences in the concentration of DHAA between the controls and the diabetic patients, and no significant differences among groups A, B, C and D.

Table 3 shows the inter-relationships between serum AA, the risk factors for atherosclerosis, and \( \Delta \text{PWV} \). The total AA value was inversely related to body mass index (BMI), atherogenic index, Apo B/Apo A-I ratio, TBARS, microalbuminuria, and \( \Delta \text{PWV} \). The AA value was also inversely related to BMI, Apo B/Apo A-I

![Figure 1. Serum ascorbic acid and dehydroascorbic acid levels in normal and diabetic groups.](image)

Data expressed as M±SD. *\( p < 0.01 \) vs. normal subjects; #\( p < 0.05 \) vs. group A.

Table 3. Correlation coefficients between serum ascorbic acid and risk factors for atherosclerosis and \( \Delta \text{PWV} \) in diabetics.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total AA</th>
<th>AA</th>
<th>DHAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>-0.274*</td>
<td>-0.273*</td>
<td>-0.076</td>
</tr>
<tr>
<td>FBG</td>
<td>-0.041</td>
<td>-0.069</td>
<td>0.102</td>
</tr>
<tr>
<td>HbA₁c</td>
<td>-0.146</td>
<td>-0.169</td>
<td>0.056</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>-0.084</td>
<td>-0.088</td>
<td>-0.003</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>-0.176</td>
<td>-0.160</td>
<td>-0.111</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>0.262</td>
<td>0.240</td>
<td>0.158</td>
</tr>
<tr>
<td>Atherogenic index</td>
<td>-0.269*</td>
<td>-0.249</td>
<td>-0.148</td>
</tr>
<tr>
<td>Apo B/Apo A-I</td>
<td>-0.288*</td>
<td>-0.285*</td>
<td>-0.087</td>
</tr>
<tr>
<td>TBARS</td>
<td>-0.280*</td>
<td>-0.288*</td>
<td>-0.041</td>
</tr>
<tr>
<td>Microalbuminuria (mg/g. Cr)</td>
<td>-0.324*</td>
<td>-0.335*</td>
<td>-0.051</td>
</tr>
<tr>
<td>( \Delta \text{PWV} )</td>
<td>-0.281*</td>
<td>-0.249</td>
<td>-0.202</td>
</tr>
</tbody>
</table>

AA, ascorbic acid; DHAA, dehydroascorbic acid; \( \Delta \text{PWV} \), the difference between the mean PWV of healthy subjects and the PWV of patients of the same age.

*\( p < 0.05 \).

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ratio, TBARS, microalbuminuria, and ΔPWV. No correlation was observed between the DHAA value and risk factors.

**DISCUSSION**

PWV testing, which is non-invasive, was used in this study to determine the severity of atherosclerosis. Patients with a high PWV value had a higher frequency of hypertension and retinopathy. Similar results were also reported by Hara et al. (20).

Numerous reports have found decreased levels of AA in diabetics (21–25), and similar results were obtained in the present study. Also, numerous cases showing reduced AA concentration inside the cells of diabetics with a high blood glucose level have been reported (22, 26–30). DHAA, the oxidized form of AA, is quickly taken up by the cell (31, 32), and is instantly reduced to AA inside the cell. Since glucose and AA share the same transport molecule on the cell membrane, the uptake of AA is impeded by glucose (28). The number of transporters on the membrane increases in the presence of insulin, and is reduced in diabetic patients (22). When foods containing AA are ingested by diabetic patients, the amount of AA that exceeds the excretion threshold of the kidneys is excreted into the urine because of the reduced uptake ability of AA in cells. However, we could not show an inverse relationship between the amount of serum AA and FBG or HbA1c in this study. For this reason, diabetic patients with marked lowering of the serum AA concentration were included considerably among them unrelated to glucose level. It was suggested that the depletion of AA was associated with diabetes and this was especially true in patients with advanced atherosclerosis, because reduced amounts of AA were observed in patients with decreased PWV values. The present study failed to take into account the lack of vitamin C intake, which might cause a decrease in the level of serum AA. However, the BMIs of the diabetic patients were higher than those of the controls, and none of the diabetic patients were on a restricted diet. A study conducted in Massachusetts reported that insulin-dependent diabetes mellitus patients were consuming adequate dietary vitamin C (30). This finding argues against the idea that the lack of vitamin C in the diet causes a decrease in the amount of serum AA.

Because of the oxidative nature of the disease, an increase in the amount of DHAA or elevation of the DHAA/AA ratio was predicted in diabetic patients before this study. However, no significant difference was observed between the patients and controls. Nevertheless, one report stated that the DHAA/AA ratio was higher in diabetics than in normal subjects (25). This may have been due to differences between the group of diabetic patients in the present study and previous reports, or possibly due to differences in the time when blood was collected. It can reasonably assumed that DHAA should not be used as an indicator of oxidation because: 1) DHAA is reduced by DHAA reductase and also reduced non-enzymatically by a thiol complex such as cysteine or glutathione and 2) DHAA is
easily and irreversibly converted to 2,3-diketogulonic acid by cleavage of the lactone ring.

AA has been reported to be a potent free radical scavenger in aqueous solution (10, 11), and is known to block the oxidation of LDL (33). Furthermore, several studies have shown that AA acts synergistically with vitamin E to block the oxidation of lipids in the cell membrane by exchanging a radical with a tocopheroxyl radical (12–15, 34, 35). This kind of relationship is also observed in human LDL (15). An oxidation test in the plasma using azo chemical compounds showed that, of all radical scavengers, AA is consumed the fastest (11). Because of the oxidative nature of the disease, it is suggested that AA is overly consumed in diabetic patients, and the reduced level of AA might lead to the development of atherosclerosis.

In addition, a decrease in the level of AA causes disruption of collagen synthesis (16, 24), and might cause cytotoxic damage in vascular endothelium. Also, AA acts as an aldose reductase inhibitor (36–38) and blocks the polyol pathway, which might slow the development of atherosclerosis.

In conclusion, our results show that the depletion of AA is observed in patients with advanced diabetic macroangiopathy.

REFERENCES


