Correlations of elevated levels of hexacosanoate in erythrocyte membranes with risk factors for atherosclerosis

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Abstract

We analyzed erythrocyte membrane C26:0 from 504 volunteers by high-performance liquid chromatography. The associations between the elevated levels of erythrocyte membrane C26:0 (0.20 or greater than 0.20%) and sex, obesity (body mass index ≥ 26.4), smoking (> 20 cigarettes per day), present illnesses and past diseases were examined with the \( \chi^2 \) test. The correlations among age and the levels of erythrocyte membrane C26:0, plasma total cholesterol, triglycerides, LDL cholesterol and HDL cholesterol were analyzed using Spearman’s correlation coefficient. Moreover, the frequencies of high levels of erythrocyte membrane C26:0 were examined in male and female subjects divided into seven age groups. The elevated levels of erythrocyte membrane C26:0 were significantly more frequent in male subjects than in females, and were closely associated with obesity, smoking, and atherosclerosis-related diseases of present illnesses. The levels of erythrocyte membrane C26:0 were highly correlated with age and the levels of plasma total cholesterol, triglycerides and LDL cholesterol, and inversely with those of HDL cholesterol. The frequency of high levels of erythrocyte membrane C26:0 in male subjects was greater than that in female subjects in all of the seven age groups. Elevated levels of erythrocyte membrane C26:0 may be closely related with atherosclerosis. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Erythrocyte membranes; Hexacosanoate; Atherosclerosis; Age

1. Introduction

Atherosclerosis is a serious health problem, and will become the chief cause of morbidity and mortality worldwide in the near future. The atherogenic process begins early in life, with a preclinical phase lasting many years, and leads to clinical complications later in life, such as myocardial infarction and stroke [1]. Therefore, presymptomatic detection of the disease is necessary to identify high-risk subjects and to apply appropriate preventive strategies.

Of the clinical tests for atherosclerosis, blood tests measuring predisposing risk factors are safe and minimally invasive. The effect of risk factors such as high levels of plasma cholesterol and low levels of high-density lipoprotein (HDL) cholesterol on the incidence of coronary disease in middle-aged people has been well established [2–6]. However, several investigators have reported that the association between risk factors and clinical coronary heart disease is weaker in the elderly [7–9].

For instance, young adults and middle-aged people with vascular disease commonly have higher total cholesterol levels, but the apparent effect of cholesterol on vascular disease wanes after the age of 50 and almost disappears after 65 [10]. Therefore, it is crucial to identify candidates of new blood risk factors for atherosclerosis.

In the present study, we studied whether erythrocyte membrane hexacosanoate (C26:0), a saturated very-long-chain fatty acid (VLFA), could be associated with well-known risk factors for atherosclerosis.
2. Methods

The subjects were 504 volunteers (324 men and 180 women), ranging in age from 25 to 65, working in the Meiji Mutual Life Insurance Company. Informed consent was obtained and information about smoking and medical histories was elicited from all of the subjects. Height and body-weight were measured to estimate the body mass index (BMI) of the subjects.

For erythrocyte membrane C26:0 assays, heparinized blood samples were obtained from fasted subjects. Erythrocyte membranes were prepared as described elsewhere [11]. The membranes were stored at −80°C until use. Total lipids were extracted from erythrocyte membranes by a modification of the method of Folch et al. [12]. The lipids in the chloroform phase were dried under a stream of nitrogen gas. Butylated hydroxytoluene, an antioxidant, was added to the chloroform and methanol used for extraction at a concentration of 5 mg/100 ml. The dried total lipid extract was hydrolyzed with 0.5 mol/l HCl in a mixture of acetonitrile and water (9:1, v/v) for 45 min at 100°C, and free fatty acids were dissolved in chloroform [13]. The fatty acids were dried under a stream of nitrogen gas and esterified with a fluorescent marker, 9-anthryldiazomethane (ADAM) (Funakoshi Drug Co., Tokyo, Japan) [14]. One millilitre of ADAM solution (5 mg ADAM in 10 ml methanol) was added to the dried fatty acids and the mixture was applied to high-performance liquid chromatography (Shimadzu LC-10AT, SCL-10A, RF-10A, DGU-3A; Shimadzu Co., Kyoto, Japan), and fatty acids were assayed as described previously [14–16]. The column was a 25 cm x 4.6 mm id stainless-steel tube prepacked with Zorbax C8 (Hewlett Packard Co., USA). The column temperature was maintained at 60°C. Fatty acids were eluted by a programmed gradient elution with acetonitrile-water (v/v, 80/20 to 100/0), and the flow rate was 1.6 ml/min. The fluorescence detector was operated at 412 nm with excitation at 365 nm. The area percentages of fatty acids were automatically calculated. We also examined the erythrocyte membrane C26:0 from nine healthy volunteers younger than 20, and all of them showed less than 0.20 in the area percentage. Therefore, erythrocyte membrane C26:0 was estimated as being high when its area percentage was 0.20 or greater.

At the same time, the plasma levels of total cholesterol, triglycerides and HDL cholesterol were measured by enzymatic procedures [17–19]. The low-density lipoprotein (LDL) cholesterol levels were calculated with the equation of Friedewald et al. [20]. The plasma levels of total cholesterol, triglycerides, HDL cholesterol and LDL cholesterol were estimated as abnormal when they were greater than 219 and 149, below 40, and greater than 139 mg/dl, respectively.

2.1. Statistical analysis

The associations of elevated levels of C26:0 in erythrocyte membranes and sex, obesity (BMI: 26.4 or more), smoking (more than 19 cigarettes per day), present illnesses and past diseases were analyzed with the χ² test.

The correlations among age and the levels of erythrocyte membrane C26:0 and four plasma risk factors for atherosclerosis (total cholesterol, triglycerides, HDL cholesterol and LDL cholesterol) were analyzed using Spearman’s correlation coefficient.

The subjects were divided into seven age groups (group 1: 25–29; 2: 30–34; 3: 35–39; 4: 40–44; 5: 45–49; 6: 50–54; 7: 55–65), and correlations between age and the frequency of high levels of erythrocyte membrane C26:0 in male and female subjects were analyzed using Spearman’s correlation coefficient.

3. Results

(1) Associations between erythrocyte membrane C26:0 and sex, obesity, smoking, present illnesses and past diseases.

Elevated levels of erythrocyte membrane C26:0 were observed in 272 of the 504 volunteers. High erythrocyte membrane C26:0 was significantly associated with male sex, obesity, and smoking (Table 1).

As for present illnesses, elevated levels of erythrocyte membrane C26:0 were also significantly associated with hypertension, hyperuricemia, diabetes mellitus and heart disease, but not with benign gastro-intestinal disease, chronic hepatitis, thyroid disease, anemia or respiratory disease (Table 1). While no subject with low levels of erythrocyte membrane C26:0 had heart disease, six of the 272 subjects with high levels of erythrocyte membrane C26:0 had heart disease (four cases of non-valvular atrial fibrillation and two cases of ischemic heart disease).

As for past diseases, none were associated with elevated levels of erythrocyte membrane C26:0 (Table 1).

(2) Correlations among erythrocyte membrane C26:0, age and four plasma lipid markers of atherosclerosis.

The levels of erythrocyte membrane C26:0 were significantly correlated with age and the levels of plasma total cholesterol, triglycerides and LDL cholesterol, and inversely correlated with those of HDL cholesterol (Table 2).

There was no significant correlation between age and the levels of plasma HDL cholesterol (Table 2). The levels of plasma total cholesterol were not correlated with those of HDL cholesterol (Table 2).

(3) Correlations between the frequency of high levels of erythrocyte membrane C26:0 and age in male and female subjects.
Table 1
Associations between erythrocyte membrane C26:0 and clinical features

<table>
<thead>
<tr>
<th></th>
<th>High RBC 26:0 (n = 272)</th>
<th>Low RBC 26:0 (n = 232)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M/F</td>
<td>4.1*</td>
<td>0.8</td>
</tr>
<tr>
<td>Obesity (%)</td>
<td>18.0*</td>
<td>5.6</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>21.7*</td>
<td>11.6</td>
</tr>
<tr>
<td><strong>Present illnesses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>8.8*</td>
<td>3.9</td>
</tr>
<tr>
<td>Hyperuricemia (%)</td>
<td>4.4*</td>
<td>0.4</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>2.6*</td>
<td>0.4</td>
</tr>
<tr>
<td>Heart disease (%)</td>
<td>2.2*</td>
<td>0</td>
</tr>
<tr>
<td>Benign GI disease (%)</td>
<td>14.7</td>
<td>12.5</td>
</tr>
<tr>
<td>Chronic hepatitis (%)</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Respiratory disease (%)</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Thyroid disease (%)</td>
<td>1.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Anemia (%)</td>
<td>0.7</td>
<td>0.4</td>
</tr>
<tr>
<td>Others (%)</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Past diseases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CVA (%)</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>Malignancy (%)</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Benign GI disease (%)</td>
<td>11.0</td>
<td>9.1</td>
</tr>
<tr>
<td>Respiratory disease (%)</td>
<td>4.4</td>
<td>6.0</td>
</tr>
<tr>
<td>Genital disease (%)</td>
<td>2.2</td>
<td>4.7</td>
</tr>
<tr>
<td>Acute hepatitis (%)</td>
<td>4.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Cholelithiasis (%)</td>
<td>2.9</td>
<td>2.2</td>
</tr>
<tr>
<td>Others (%)</td>
<td>5.1</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* *, $P<0.05$. Values are ratios in ‘M:F’ and incidences in others. High RBC 26:0, 0.20 or more than 0.20 of area percentage of erythrocyte membrane C26:0; Low RBC 26:0, less than 0.20 of area percentage of erythrocyte membrane C26:0; M:F, the ratio of males to females. Malignancy: post-operative state of malignant tumors; benign GI disease: benign gastro-intestinal disease.

There were significant correlations between the frequency of high levels of erythrocyte membrane C26:0 and age in both male and female subjects (Fig. 1). High levels of erythrocyte membrane hexacosanoate were more frequent in male subjects than in female subjects in all of the seven age groups (Fig. 1).

4. Discussion

Hexacosanoate is a saturated VLFA and a minor fatty acid component in human tissues. However, this fatty acid has been used as a diagnostic marker for peroxisomal disorders including adrenoleukodystrophy [21–23], which involves the abnormal metabolism of VLFA resulting in an accumulation of VLFA in tissues.

We previously investigated hexacosanoate from erythrocyte membranes, lymphocytes and blood plasma in adrenoleukodystrophy, and found an overlapping in the level of hexacosanoate between adrenoleukodystrophy patients and aged controls only in erythrocyte membranes, against our expectation [23]. This finding provoked us to study erythrocyte membrane hexacosanoate with respect to age-related pathologies.

The present study showed that the levels of erythrocyte membrane hexacosanoate were associated with

Table 2
Correlations among age and the levels of erythrocyte membrane C26:0 and plasma total cholesterol, triglycerides, HDL cholesterol and LDL cholesterol in 504 volunteers

<table>
<thead>
<tr>
<th>Age</th>
<th>RBC 26:0</th>
<th>TC</th>
<th>TG</th>
<th>HDL-c</th>
<th>LDL-c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>−</td>
<td>0.379*</td>
<td>0.402*</td>
<td>0.223*</td>
<td>−0.072</td>
</tr>
<tr>
<td>RBC26:0</td>
<td>0.379*</td>
<td>−</td>
<td>0.281*</td>
<td>0.433*</td>
<td>−0.257*</td>
</tr>
<tr>
<td>TC</td>
<td>0.402*</td>
<td>0.281*</td>
<td>−</td>
<td>0.401*</td>
<td>0.004</td>
</tr>
<tr>
<td>TG</td>
<td>0.223*</td>
<td>0.433*</td>
<td>−</td>
<td>−</td>
<td>−0.550*</td>
</tr>
<tr>
<td>HDL-c</td>
<td>−0.072</td>
<td>−0.257*</td>
<td>0.004</td>
<td>−0.550*</td>
<td>−</td>
</tr>
<tr>
<td>LDL-c</td>
<td>0.393*</td>
<td>0.206*</td>
<td>0.798*</td>
<td>0.192*</td>
<td>−0.165*</td>
</tr>
</tbody>
</table>

* Values are Spearman’s correlation coefficients. *, $P<0.01$; RBC 26:0, erythrocyte membrane C26:0; TC, plasma total cholesterol; TG, plasma triglycerides; HDL-c, HDL cholesterol; LDL-c, LDL cholesterol.
those of the well-known risk factors for atherosclerosis, i.e. plasma total cholesterol, triglycerides, LDL cholesterol, HDL cholesterol and age. Moreover, it was shown that obesity, smoking and atherosclerosis-related diseases were associated with high levels of erythrocyte membrane hexacosanoate. These results suggest that increased levels of erythrocyte membrane hexacosanoate are closely related to atherosclerosis.

High levels of erythrocyte membrane hexacosanoate were more frequent in male subjects than in female subjects, especially in younger ages. This finding suggests that levels of erythrocyte membrane hexacosanoate can be affected by sex hormones, though its mechanism remains unclear.

What causes an accumulation of hexacosanoate in erythrocyte membranes? Is hexacosanoate accumulated in erythrocyte membranes exogenously or endogenously? We cannot clearly answer these questions at present. However, we can speculate that the elevation of erythrocyte membrane hexacosanoate levels reflects the decreased functions of peroxisomes, intracellular organella. Hexacosanoate is oxidized by peroxisomal β-oxidation [24], and age-related changes in peroxisomal fatty acid oxidation activity were characterized in rodents [25,26]. Besides, it is reported that desorption of hexacosanoate from membranes is slower than that of shorter chain fatty acids [27]. Therefore, a decrement of peroxisomal functions caused by various factors, exogenous or endogenous, may result in the elevation of hexacosanoate levels of erythrocyte membranes and cell membranes of other tissues.

It can be speculated that the accumulation of hexacosanoate in cell membranes inevitably reduces the fluidity of the membranes. This reduced membrane fluidity can cause various abnormalities in cell membrane functions resulting in abnormalities of intracellular metabolism. Whitcomb and colleagues reported that saturated VLFA (C26:0 and C24:0) suppressed the adrenocorticotropic responsiveness of cultured human adrenocortical cells, which suggests that hexacosanoate can affect ACTH receptor functions [28]. Their results may suggest a close relationship between saturated VLFA and membrane receptor functions.

Hexacosanoate is contained more in tightly-bound fatty acids than loosely-bound fatty acids in membrane proteins [29]. Tightly-bound fatty acids are not removed by exhaustive extraction with organic solvents, phospholipase A2 treatment, or sodium dodecyl sulfate, but are released by refluxing with hot methanolic HCl or in part by mild alkaline hydrolysis [29,30]. This fact suggests that tightly-bound fatty acids are distinguished from other membrane fatty acids (loosely-bound fatty acids) and may be covalently attached to membrane proteins [30]. Therefore, an elevation of hexacosanoate levels in the tightly-bound fatty acids can crucially affect membrane receptor functions, and may be related to atherogenic processes in vascular endothelial cells, smooth muscle cells and so on.

If so, reducing membrane hexacosanoate may result in inhibiting the atherosclerotic processes. Several agents were reported to reduce hexacosanoate. For instance, monounsaturated long-chain fatty acids (e.g. eicosenoic acid and docosenoic acid) are known to inhibit the biosynthesis of VLFA [31]. These fatty acids may prevent atherosclerotic diseases. Furthermore, Singh et al. reported that lovastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor suppressing cholesterol synthesis, markedly reduced accumulated VLFA in patients with adrenoleukodystrophy [32]. This finding suggests that some lipid-lowering agents may inhibit atherosclerotic processes by reducing the level of membrane hexacosanoate. In conclusion, our data suggest that erythrocyte membrane hexacosanoate may be associated with risk factors for atherosclerosis. However, further investigations are necessary to clarify whether or not the elevation of erythrocyte membrane hexacosanoate levels is a new risk factor for atherosclerosis and how the hexacosanoate accumulation in cell membranes participates in the atherosclerotic processes.

References