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Alterations of Fatty Acid Composition of Erythrocyte Membrane in Type 2 Diabetes Patients with Diabetic Retinopathy

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Purpose: To investigate the relationship between the erythrocyte membrane fatty acid components and diabetic retinopathy in patients with type 2 diabetes mellitus.

Methods: The study was performed in 40 patients. Thirty of the 40 were type 2 diabetic patients classified into three groups according to Early Treatment Diabetic Retinopathy Study Group (ETDRS) criteria: 10 with mild–moderate nonproliferative retinopathy (group 1), 10 with moderate–severe nonproliferative retinopathy (group 2), and 10 with proliferative retinopathy (group 3). Ten age- and sex-matched healthy nondiabetic individuals were selected as controls. We examined the fatty acid composition of the erythrocyte membrane by a gas chromatographic method.

Results: In patients with nonproliferative diabetic retinopathy, we found statistically significant decreases in palmitic and stearic acids, and statistically significant increases in oleic, linoleic, behenic, and lignoceric acids, while arachidic and arachidonic acids remained unchanged. Except for the increase in arachidic acid, the results were similar to those in the proliferative retinopathy patients.

Conclusions: The fatty acid component of the erythrocyte membrane alters in type 2 diabetes mellitus. Prospective studies are needed to evaluate the relationship between the free-fatty acid composition of erythrocytes and diabetic retinopathy. **Jpn J Ophthalmol 2003;47:551–556** © 2003 Japanese Ophthalmological Society

Key Words: Diabetes mellitus, erythrocyte membrane, fatty acids, retinopathy.

Introduction

Hemodynamic changes are known to play an important role in the pathogenesis of diabetic retinopathy: increased blood viscosity, increased erythrocyte aggregation, altered erythrocyte permeability, and increased adhesion of erythrocytes to endothelial cells.[1–4] In diabetic patients, a reduction of erythrocyte deformability and an increase of whole-blood viscosity were correlated with microangiopathy.[5] However, blood flow abnormalities, the factors that mediate them, and their role in the development of proliferative retinopathy are still unclear.[6] Focal areas of capillary closure and nonperfused capillaries appear to develop relatively early after the onset of diabetes. It was postulated that hypoxic and ischemic retinas adjacent to such areas release vasoactive agents and growth factors that increase blood flow and initiate vasoproliferative changes. The exact mechanism responsible for the capillary closure and nonperfusion, and whether these changes are preceded and caused by blood flow changes, are not fully understood.[7]

The membrane surrounding the erythrocyte forms a boundary between the interior of the cell and the plasma surrounding it, and serves as a barrier to help maintain the interior of the red cell. It must be insoluble in aqueous solution; not surprisingly, then, approximately one half of the mass of the human erythrocyte membrane consists of lipid, largely arranged as a bilayer.[8] In addition to serving as a barrier, the membrane contains pumps and channels for the movement of sodium, potassium, and calcium, and it facilitates the transport of glucose and other small molecules. It is also responsible for the biconcave shape and basic structural integrity of the erythrocyte.

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These functions mentioned above are performed by the presence of membrane proteins.[9]

The changes of erythrocyte membrane properties induced by high levels of free-fatty acids or aldehydes, which can be produced in membranes during peroxidative processes, may be responsible for some long-term complications in a number of diseases, such as diabetes mellitus.[10] It has also been suggested that the ability of red blood cells to change their shape (deformability) is decreased in diabetic patients. Such an impairment of the red blood cells' deformability might be another contributing factor to the reduction of blood flow in the capillaries.[7]

The differences of results in the literature led us to further investigations of the lipid composition of erythrocyte membrane in diabetes, paying special attention to the selection of the patients and the quality of the analytic methods. The aim of this study was to investigate the free-fatty acid composition of erythrocyte membranes of patients with diabetic retinopathy and thus, to evaluate the possible contributing factors leading to the development of diabetic retinopathy.

Materials and Methods

We studied 30 type 2 diabetes mellitus (DM) patients and 10 age-and sex-matched healthy nondiabetic controls in this study. We obtained approval from the ethics committee of Atatürk University Medical School and all procedures followed the tenets of the Declaration of Helsinki. Written informed consent was given by the patients included in the study. The patients were divided into three groups according to the severity of diabetic retinopathy. Each group consisted of 10 patients. Their mean ages were 53.8, 54.8, and 57.3 years in groups 1, 2, and 3, respectively. The mean age was 54.2 years in the healthy nondiabetic control group. None of the subjects in this study had hypertension and no one was smoking, taking alcohol or any lipid-lowering drug.

After complete ophthalmologic examinations through dilated pupils, with fundus fluorescein angiography if clinically indicated, the diabetic patients were classified according to the retinopathy severity criteria of the Early Treatment Diabetic Retinopathy Study Group (ETDRS).[11] This classification was based on the following findings.

Group 1: mild–moderate nonproliferative retinopathy; microaneurysms, intraretinal hemorrhages (less than four quadrants and mild to moderate degree), hard exudates, macular edema, anomalies in foveal avascular zone

Group 2: moderate–severe nonproliferative retinopathy, cotton wool spots, intraretinal hemorrhages (four quadrants and mild to moderate degree), venous beading, and intraretinal microvascular anomalies

Group 3: proliferative retinopathy; neovascularisations on disk, neovascularisation elsewhere, preretinal hemorrhages, vitreous hemorrhage, tractional retinal detachment, neovascularisation at iris, and/or anterior chamber angle

After overnight fasting by the patients, 10 mL of blood was drawn by venipuncture into heparinised glass tubes. Red cells were separated from plasma by centrifugation at 576 *g* for 10 minutes. The plasma and buffy coat were carefully removed from red blood cells using a glass pipette. Packed erythrocytes were lysed in 5 mL of ice-cold deionised water were added to the erythrocytes to induce haemolysis. The sample was then centrifuged at 48,000 *g* for 1 hour to separate the haemolysate from the pellet of red blood cell membranes. Then the pellets of red blood cells were washed until the precipitate of membrane particles became uncoloured. The precipitate containing membrane particles was treated with 0.05 M Tris-SO_4 (pH 7.4) and 1% Triton-X 100 solutions for 4 hours in the ultrasonic dismembrator (550 Sonic Dismembrator; Fischer Scientific, Pittsburgh PA, USA). It was centrifuged at 48,000 *g* for 30 minutes, and then was washed by 0.9% NaCl suspension to remove the remnants of Tris-SO_4 and Triton-X 100.[12] Thus, lipids were separated from proteins.

Total lipids were extracted from erythrocyte membrane suspension in a modification of the method of Herbert and Morris.[13] Phospholipids were separated from other lipids by thin layer chromatography (TLC) on glass plates (4 mm thickness) coated with a 0.5-mm layer of Silica Gel 60 G (Merck). One millilitre of acetate buffer (0.5 M, pH 4) was added to the resulting lipid component. To this mixture, 10 mL of phospholipase enzyme, which catalyzes the hydrolysis of ester linkages in phospholipids, was added.[14] Hydrolysis was carried out by ultrasonification plus phospholipase B. In order to test whether hydrolysis of phospholipids is achieved or not, 1×5 cm commercially available TLC plates were utilised. Phospholipid spots seen before hydrolysis were not detected, thus indicating that both of the ester linkages had been hydrolysed. After that, the samples were mixed with 1 mL of chloroform. The chloroform phase obtained was put into special chromatography jars. Again, 1 mL of chloroform was added and the tube was shaken up and down two to three times. Then, chloroform was transferred into a glass jar and dried under nitrogen. Two-hundred microlitres of chloroform was added and covered with a Teflon-coated aluminium lid and stored at -70ºC in deep freeze until assayed.

The samples were analysed by capillary gas chromatography (GC 6000 Vega Series 2; Carlo Erba Instruments, Milano, Italy) fitted with a gas column (15 m × 0.53 mm i.d.) (Nucol). The peaks obtained were evaluated by comparison with available standard specimens (Merck).

Statistical Analysis

The results in patients and healthy nondiabetic controls were evaluated statistically by the Student *t*-test and multivariate analysis of variance (MANOVA using STATISTICA Version 5.0). In multivariate analysis, independent factors were controls and group 1, group 2, group 3, respectively, consisting of 10 patients in each group. Dependent factors were fatty acids determined in the erythrocyte membranes for all patients. Multiple regression analysis (STATISTICA Version 5.0) was used to show the correlations between the fatty acid composition of controls and for each group of patients with diabetic retinopathy. Mean values were given with their standard deviation of the mean. The criterion of significance is a P value $<.05$ for both the Student *t*-test and the multivariate analysis.

Results

Each patient and the healthy nondiabetic control groups were age-and sex-matched. Table 1 exhibits the mean age of each subgroup of patients, as well as the duration of diabetes, laboratory data including erythrocyte counts, HbA_{1C}, cholesterol, and triglyceride levels of the population studied. In the chromatograms of fatty acids (not shown), significant peaks were detected in palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), behenic acid (22:0), lignoceric acid (24:0), arachidic acid (20:0), and arachidonic acid (20:4). The fatty acid distribution as a percentage of the total weight of the fatty acids is presented in Figure 1 and Table 2. The fatty acid composition of the groups was compared with that of the control group. Table 2 also shows the statistical evaluation of the free-fatty acid composition between the healthy nondiabetic controls and each of the groups.

The results indicated that palmitic and stearic acids were decreased, and oleic, linoleic, behenic and lignoceric acids were increased in all patient groups (groups 1, 2, and 3). In general, arachidic and arachidonic acids remained unchanged except that arachidic and arachidonic acid increased in group 3 and group 1, respectively. The most significant increases were observed in lignoceric, oleic, behenic, and linoleic acid levels ($P < .001$; Student *t*-test, in all groups). The most significant decreases were found proportionally with the degree of diabetic retinopathy at the levels of palmitic and stearic acids ($P < .001$; Student *t*-test, in all groups) (Table 2). The levels of arachidonic acid remained nearly at the same level in all groups. Arachidic acid levels in groups 1 and 2 also seemed unchanged but it increased in group 3. The palmitic and stearic acid levels were negatively correlated with linoleic, oleic, and lignoceric acid levels, and there was a strong correlation between the control and patient groups ranging in group 1: 0.97, group 2: 0.94, and group 3: 0.90, according to multiple regression analysis. The decrease in the correlations for the diabetic patient groups shows that the changes in patients' fatty acid profiles are related with the severity of diabetic retinopathy. Figure 1, which also reflects the correlations between controls and patients with diabetic retinopathy, shows the fatty acid distribution as a percentage of the total weight of the fatty acids. MANOVA also showed a statistically significant difference between controls and group 1 ($P < .005$), group 2 ($P < .00002$), and group 3 ($P < .00003$), respectively.

Table 1. Clinical Characteristics of Three Groups of Patients Classified by Retinopathy*

Subjects	Group 1	Group 2	Group 3	Control
No. in each group	10	10	10	10
Age range (yr) (mean)	43–74 (53.7)	49–71 (54.8)	48–67 (57.3)	45–68 (54.2)
Female/male	7/3	5/5	6/4	5/5
Duration of DM† (yr) (mean)	1–15 (7.9)	4–18 (10.2)	5–24 (13.2)	–
Glycated haemoglobin (%) (HbA_{1C}) (mean ± SD)	8.81 ± 2.48	8.80 ± 1.09	11.94 ± 2.60	4.38 ± 1.27
Triglyceride (mg/dL)	187.07 ± 74.30	234 ± 145.24	146.30 ± 52.41	179.60 ± 57.38
Cholesterol (mg/dL)	182.0 ± 51.51	183.18 ± 52.95	225.14 ± 66.34	174.3 ± 42.32
Erythrocyte count ($10^6/\mu L$)	4.95 ± 0.55	4.14 ± 0.91	3.49 ± 0.96	5.38 ± 0.55

*(Group 1: mild–moderate nonproliferative retinopathy, group 2: moderate–severe nonproliferative retinopathy, group 3: proliferative retinopathy) and healthy nondiabetic control subjects.

†DM: diabetes mellitus.

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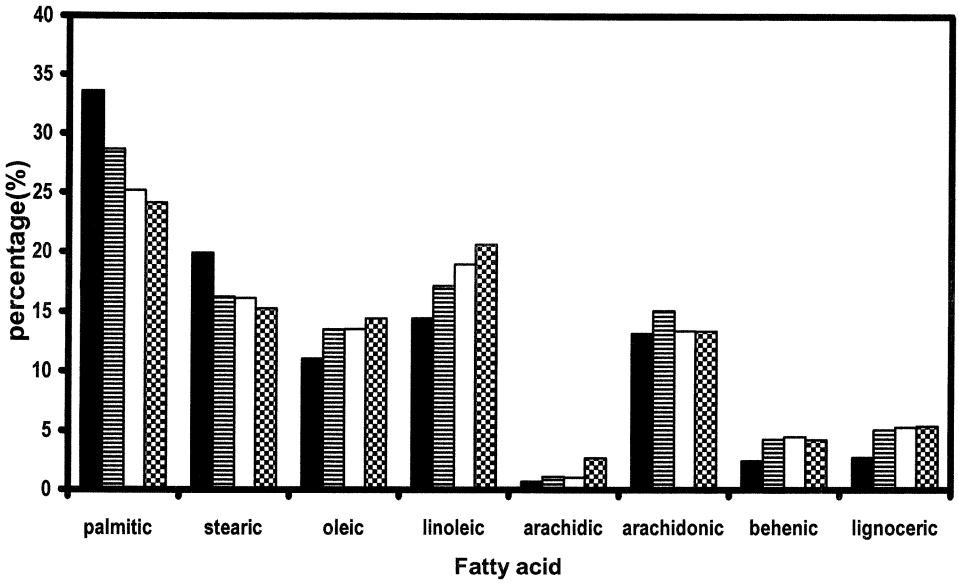

Figure 1. The fatty acid distribution as a percentage of the total weight of the fatty acids: (Group 1: Mild-moderate nonproliferative retinopathy, Group 2: Moderate-severe nonproliferative retinopathy, Group 3: Proliferative retinopathy). ■: Control, ▤: Group 1, □: Group 2, ▩ Group 3.

Discussion

Alterations of the flow characteristics within the microcirculation and macrocirculation are thought by many authors to be related to the development of the vascular complications of diabetic retinopathy. It has been suggested that alterations seen in erythrocytes of diabetic patients may be partly due to the modification of the membrane lipid composition and/or membrane fluidity. Because it is reasonable to assume that the lipid composition partially determines membrane viscosity, any significant changes in lipid composition which affect the membrane's internal microviscosity might be expected to have some effect on the flexibility of the whole cell. Indeed, the membrane fluidity is mostly influenced by factors such as the relative concentration of cholesterol and phospholipid, the composition in phospholipids, and the length and structure of fatty acids.[15]

Normally, the total fatty acid profile of the erythrocyte membrane consists of 41% palmitic acid, which is the greatest one in amount.[16] Thus, any change in the amount of palmitic acid can be important in the total fatty acid profile. We found that palmitic and stearic acids in all diabetic groups decreased significantly ($P < .001$; Student *t*-test) compared with the healthy nondiabetic controls in proportion to the degree of diabetic retinopathy. In addition, oleic, linoleic, behenic, and lignoceric acids increased ($P < .001$; Student *t*-test, in all diabetic groups) almost proportionally with the degree of diabetic retinopathy (Figure 1).

Table 2. Distribution and Statistical Evaluation of the Free-fatty Acid Composition Between the Healthy Nondiabetic Controls (C) and Each of the Diabetic Groups*

	Control		Group 1		Group 1-C	Group-2		Group 2-C	Group 3		Group 3-C
Fatty Acids	Mean	SD	Mean	SD	*P*-Value	Mean	SD	*P*-Value	Mean	SD	*P*-Value
16:0	33.88	2.89	28.66	2.13	<.001	25.16	1.96	<.001	24.13	2.36	<.001
18:0	20.02	2.38	16.25	1.80	<.001	16.14	1.76	<.001	15.25	1.43	<.001
18:1	10.08	2.82	13.51	0.92	<.05	13.56	1.91	<.05	14.42	1.91	<.05
18:2	13.99	1.75	17.15	1.44	<.001	18.95	1.78	<.001	20.62	2.01	<.001
20:0	0.70	0.48	1.09	0.43	†	1.04	0.30	†	2.67	0.09	<.001
20:4	13.09	1.75	15.10	2.30	<.05	13.39	1.83	†	13.36	1.90	†
22:0	2.09	1.16	4.28	1.07	<.001	4.48	1.43	<.001	4.20	1.10	<.001
24:0	2.28	0.95	5.06	1.17	<.001	5.06	1.02	<.001	5.38	0.78	<.001

*Group 1: mild–moderate nonproliferative retinopathy, group 2: moderate–severe nonproliferative retinopathy, group 3: proliferative retinopathy.
†$P > .05$; statistically not significant.

Previous studies that investigated the phospholipid and fatty acid composition of erythrocytes in diabetic patients yielded conflicting results. Taylor et al[17] found significant decreases in stearic acid and arachidonic acid and significant increases in palmitic acid in erythrocytes from diabetic patients. We obtained similar results for stearic acid concentration; whereas arachidonic acid levels were unchanged and the decrease of palmitic acid in our study was different from that of Taylor et al. They proposed that impaired metabolic control associated with diabetes mellitus might interfere with the maintenance of fatty acid profiles in erythrocyte membranes against the concentration gradients in plasma. However, Faas et al[18] reported increased levels of linoleic acid. The same result was found in this study. Also, Gangyi et al[19] investigated the relationship between erythrocyte membrane fluidity and fatty acid components in diabetic retinopathy. They reported that the content and constituent percentage of erythrocyte membrane arachidonate ($C_{20,4n-6}$) were significantly lower in the diabetic patients than in the controls, whereas it remained unchanged in our study. They found higher membrane microviscosity in diabetic patients with diabetic retinopathy than in those without. The microviscosity values positively correlated with palmitate ($C_{16,0}$) and stearate ($C_{18,0}$) levels in the erythrocyte membrane and negatively correlated with arachidonate ($C_{20,4n-6}$) and docosahexaenote ($C_{22,6n-3}$) in patients with type 2 DM. The impact of the severity of diabetic retinopathy on the changes in erythrocyte fatty acid profiles was investigated in our study and not in the others. The fatty acid profiles of the patients change proportionally with the severity of diabetic retinopathy, as is clearly seen in Figure 1. Multivariate analysis, supporting the results of the Student *t*-test, also showed that the fatty acid profiles of the controls and the patients with diabetic retinopathy were significantly different.

Some authors described an increase of the cholesterol/phospholipid ratio[20] whereas others reported no such alteration.[21] An increased concentration of the saturated fatty acids was described by one group,[22] and another team showed an increase of the linoleic acid level.[23] A significant decrease in the amounts of stearic and arachidic acids in the erythrocytes of diabetic patients was reported, whereas the abundance of the other analysed fatty acids remained unchanged.[17] We found similar results for linoleic acid and stearic acid levels. Furthermore, arachidic and arachidonic acid levels can be accepted as remaining unchanged in our study. Freyburger et al[15] demonstrated a slight but significant increase in the phosphatidylethanolamine/phosphatidylserine ratio, and an increase in phosphatidylinositol and phosphatidic acid in diabetic patients, whereas they showed no significant differences in their fatty acid contents.

Lapshina et al[10] studied the state of the membrane by using the activity of membrane-bound acetyl-cholinesterase (AChE). They found that free-fatty acids and corresponding aliphatic aldehydes induced an inhibition of membrane-bound AChE and effectively decreased the bulk lipid and protein-bound lipid microviscosity. They also showed that palmitic acid produced a noncompetitive inhibition of membrane-bound AChE. It is proposed that fatty acids and related compounds perturb the lipid bilayer and disturb the protein–lipid complementarity of the human erythrocyte membrane. Modification of the lipid composition of the membrane can significantly influence both the mechanical flexibility of the red blood cell and the passive cation permeability of the membrane. However, the aliphatic chain elongation of long-chain fatty acids containing more than 12 carbon atoms diminished the inhibitory effect, which may be related to the decreased solubility of the fatty acid in an aqueous suspension.

In diabetic microangiopathy, the capillary diameter decreases even further because of the thickening of the basal membrane and the accumulation of metabolites on the endothelial surface of the diabetic eye.[24] The exact mechanism responsible for the capillary closure and nonperfusion, and whether these changes are preceded and caused by blood flow changes, is not fully understood. Alteration in the fatty acid profile may play a role in reducing whole-cell deformability. Therefore, the capability of the erythrocyte to enter and pass through small capillaries is reduced. The end result can be vascular occlusion and ischemia contributing to the development of diabetic retinopathy. However, phospholipids and nonesterified cholesterol account for more than 95% of the total lipid within the membrane. In addition, small amounts of glycolipids, glycerides, and free-fatty acids are also present.[9] Therefore, an alteration in the fatty acid profile may not be the only reason influencing the membrane microviscosity, membrane flexibility, and whole-cell deformability. It might be suggested that such alterations in the fatty acid profile may be contributing to the cause of diabetic retinopathy.

Conclusion

In our patients with nonproliferative diabetic retinopathy, the fatty acid profiles clearly showed that palmitic and stearic acid levels were decreased, whereas oleic, linoleic, behenic, and lignoceric acids were increased. Arachidic and arachidonic acids remained unchanged. In addition, except for the slight increase in arachidic acid, the results were similar in proliferative retinopathy. Prospective studies are needed to confirm the relationship of free-fatty acid compositions as a marker of progression in diabetic retinopathy.

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