Alterations in Lymphocyte Membrane Protein Content and Increased Lymphocyte Rigidity in Cats with Diabetes Mellitus

Diabetes Mellituslu Kedilerde Lenfosit Membran Protein İçeriğinde Değişiklikler ve Artmış Lenfosit Rijiditesi

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ABSTRACT Objective: In a previous study we showed that red cell deformability was decreased in cats with non-insulin dependent diabetes mellitus (NIDDM) and it was associated with abnormalities in various rheological parameters (blood and plasma viscosity, erythrocyte deformability, hemotological parameters) as well as alterations in red cell membrane proteins. On the other hand, there is limited data concerning the contribution of lymphocyte membrane protein abnormalities and increased lymphocyte rigidity to impaired blood rheology in diabetes. In the present study, we aimed to investigate lymphocyte deformability and alterations in lymphocyte membrane proteins in cats with NIDDM. **Material and Methods:** In this regard, we analyzed lymphocyte deformability in 10 cats with non-insulin dependent diabetes mellitus (male/female: 5/5, mean age: 3.08 ± 1.39 years, mean weight: 3.26 ± 1.25 kg.) and 10 healthy controls (male/female: 6/4, mean age 5.7 ± 1.91 years, mean weight: 4.56 ± 1.16 kg.) by using the microfilter technique. We also assessed membrane protein content by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In addition, blood levels of glucose, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and trygliceride were determined. **Results:** We found that lymphocyte rigidity (∓) was significantly increased in cats with NIDDM compared to controls (1.215 ± 0.532 vs. 0.265 ± 0.42; p<0.01). Blood (3.8 ± 0.1 vs 4.4 ± 0.2; p< 0.001) and plasma viscosity (1.2 ± 0.1 vs. 1.4 ± 0.1; p< 0.01) were significantly higher in the diabetic group. Blood levels of glucose (99.8 ± 21 mg/dL vs. 177.9 ± 53.2 mg/dL; p< 0.001), total cholesterol (106.4 ± 15.6 mg/dL vs. 183.4 ± 57.8 mg/dL; p< 0.001), LDL (12.1 ± 5.3 mg/dL vs. 24.7 ± 14.6 mg/dL; p< 0.02) and HDL (78.7 ± 9.5 mg/dL vs. 133.4 ± 44.9 mg/dL; p< 0.001) were significantly higher in the diabetic group. SDS-PAGE revealed that the band which corresponds to the protein with a weight of 37 kDA had disappeared in cats with NIDDM. **Conclusion:** We suggest that the observed abnormalities in membrane proteins may play a role in reduced lymphocyte deformability associated with diabetes mellitus and may have a role in increased blood viscosity.

Key Words: Diabetes mellitus; lymphocytes; hemorheology; electrophoresis, polyacrylamide gel

ÖZET Amaç: Daha önceki bir çalışmada, insülinden bağımsız diabetes mellituslu (NIDDM) kedilerde eritrosit şekil değiştirebilirliğindeki azalmanın membran proteinleri ve çeşitli hemoreolojik parametrelerdeki (kan ve plazma viskozitesi, eritrosit deformabilitesi, hematolojik parametreler) anormalliklerle ilişkili olduğunu göstermiştik. Diğer taraftan, bozulmuş lenfosit membran proteinlerinleri ve artmış lenfosit rijiditesinin diyabetteki bozulmuş kan akışkanlığına olan katkısı hakkında sınırlı bilgi bulunmaktadır. Bu çalışmada, NIDDM'li kedilerde lenfosit şekil değiştirebilirliği ve lenfosit membran proteinlerindeki değişiklikleri araştırmayı amaçladık. **Gereç ve Yöntemler:** Çalışmanın amacı doğrultusunda 10'u NIDDM'li (erkek/dişi: 5/5; ortalama yaş: 3.08 ± 1.39 yıl; ortalama ağırlık: 3.26 ± 1.25 kg.); 10'u da sağlıklı kontrol (erkek/dişi: 6/4; ortalama yaş: 5.7 ± 1.91 yıl; ortalama ağırlık: 4.56 ± 1.16 kg) olmak üzere toplam 20 kedide mikrofiltre tekniği ile lenfosit şekil değiştirebilirliğini analiz ettik. Aynı zamanda sodyum dodesil poliakrilamid jel elektroforezi (SDS-PAGE) ile membran proteini içeriğini değerlendirdik Ayrıca kan glukoz, total kolesterol, yüksek dansiteli lipoprotein (HDL), düşük dansiteli lipoprotein (LDL), çok düşük dansiteli lipoprotein (VLDL) ve trigliserid düzeyleri tayin edildi. **Bulgular:** Sağlıklı kontrollere göre NIDDM'li kedilerde lenfosit rijiditesini artmış bulduk (1.215 ± 0.532 ve 0.265 ± 0.42; p< 0.01). Hem kan viskozitesi (4.4 ± 0.2 ve 3.8 ± 0.1; p< 0.001) hem de plazma viskozitesi (1.4 ± 0.1 ve 1.2 ± 0.1; p< 0.01) diyabetik kedilerde kontrollerden anlamlı olarak yüksekti. Kan glukoz (177.9 ± 53.2 mg/dL ve 99.8 ± 21 mg/dL; p< 0.001), total kolesterol (183.4 ± 57.8 mg/dL ve 106.4 ± 15.6 mg/dL; p< 0.001), LDL (24.7 ± 14.6 mg/dL ve 12.1 ± 5.3 mg/dL; p< 0.02) ve HDL (133.4 ± 44.9 mg/dL ve 78.7 ± 9.5 mg/dL; p< 0.001) düzeyleri diyabetik grupta anlamlı olarak artmış bulundu. NIDDM'li kedilerin SDS-PAGE'sinde, 37 kDA ağırlığına denk gelen protein bandının kaybolduğu saptandı. **Sonuç:** Membran proteinlerinde görülen bozuklukların diyabetle ilişkili azalmış lenfosit şekil değiştirebilirliğinde ve artmış kan viskozitesinde rol oynayabileceğini düşünmekteyiz.

Anahtar Kelimeler: Diabetes mellitus; lenfositler; kan akımı bilimi; elektoforez, poliakrilamid jel

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Diabetes mellitus is a chronic metabolic disease characterized mainly by disturbed glucose metabolism, where the most serious complications are associated with micro- and macrovascular angiopathy. In particular, it has been well established that microvascular involvement contributes to the development of retinopathy, nephropathy, heart disease and neuropathy.[1] On the other hand, in recent years there has been a great interest in the possible role of abnormal blood rheology on the pathogenesis of microvascular complications. It has been reported that several parameters affecting blood flow mechanics, including blood viscosity and erythrocyte deformability are altered in patients with diabetes mellitus.[2-5] Although there is a clear and linear relation between blood glucose and microvascular complications, it was suggested that abnormal rheology might also result in further worsening of the tissue hypoxia associated with diabetic microangiopathy. However, there is limited data concerning the contribution of cell membrane protein abnormalities to the increased red cell rigidity and blood rheology.[6] We have previously shown the presence of erythrocyte membrane protein abnormalities on sulfate polyacrilamide gel electrophoresis (SDS-PAGE) in a population of non-insulin dependent diabetic cats.[7] In the present study, as an attempt to extend our observations to lymphocytes, we analysed lymphocyte rigidity and assessed lymphocyte membrane protein content by SDS-PAGE in cats with NIDDM.

MATERIAL AND METHODS

The study group involved 10 cats with non-insulin dependent diabetes mellitus (male/female: 5/5, mean age: 3.08 ± 1.39 years, mean weight: 3.26 ± 1.25 kg), whereas the control group consisted of 10 (male/female: 6/4, 5.7 ± 1.91 years, mean weight: 4.56 ± 1.16 kg) healthy cats. All the cats were diagnosed with type II diabetes mellitus in the outpatient clinic of Istanbul University Veterinary Faculty, Department of Internal Diseases. Venous blood samples were obtained from each subject in the study and control groups. We analyzed lymphocyte deformability and lymphocyte membrane proteins.

SEPARATION OF LYMPHOCYTES FROM BLOOD

Blood was collected by venipuncture and put into EDTA-treated evacuated tubes. Three mls of HISTAPAQUE-1077 was added to each 15 ml conical centrifuge tube. Three mililiters whole blood was added onto the HISTAPAQUE-1077, and centrifuged at 400 x g for exactly 30 minutes at room temperature. After centrifugation, the upper layer was carefully aspirated with a Pasteur pipet into 0.5 cm of the opaque interface containing mononuclear cells. The upper layer was discarded. The opaque interface was carefully transferred, with a Pasteur pipet, into a clean conical centrifuge tube. Interfaced cells were harvested and then resuspended in PBS and then lysed by freezing and dissolving three times. After centrifugation at 3000 rpm for 10 minutes, the supernatant was taken.[8]

SDS-PAGE OF LYMPHOCYTE CELL MEMBRANE

We performed SDS-PAGE by a mini-gel system using a 10% gel and a discontinuous buffer system (Bio-Metra, Minigel-Twin, Göttingen, Germany). Samples were diluted with an equal volume sample buffer (125 mM Tris-HCl buffer, pH: 6.8, containing 2% SDS, 0.05% 2- mercaptoethanol and bromophenol blue). The gels were stained with Coomassie Brillant Blue R-250 in methanol/acetic acid. Finally, the gels were destained until the protein bands were clear. A mixture of molecular mass standards was also run: bovine serum albumin (66 kDa), ovalbumin (45kDa), carbonic anhydrase (29 kDa) and bovine trypsinogene (24 kDa) were used as standards (Sigma MW-SDS-70, MO, USA). The electrophoretic pattern of lymphocyte membranes on SDS-PAGE in cats with NIDDM were compared with those of healthy controls.[9]

DETERMINATION OF HEMORHEOLOGICAL PARAMETERS

Lymphocyte deformability was examined with microfilter technique,[10] which used the pressure required to force cells to pass through a polycarbonate filter having a pore diameter of 5 mm (Millipore, Isopore® membrane filter TMTPO2500, Lot no R1HN11485 Hertfordshire, Ireland) as the index of deformability. The cell suspension was pumped into the filter at a constant flow rate of 0.5

ml/min for 5 minutes at room temperature. The filtration pressure was measured on the upstream side of the filter with a pressure transducer (Gould, Model TMP400, P231D, USA) connected to an amplifier and a recorder (Nihon Kohden RM 6000, Tokyo, Japan).

For erythrocyte deformability, blood was filtered through cotton wool to remove leukocytes and platelets and then the eythrocytes were washed three times in PBS. The hematoccrite level was determined with the microhematocrite method and the samples were resuspended to obtain a final hematocrit value of 5%. Erthrocyte deformability was then assessed similar to lymphocyte deformability.[10]

Blood and plasma viscosity of blood samples were determined with Wells-Brookfield LUT cony-plane rotatory viscometer (MAO-202072 Engineering Laboratories, Stoughton, USA) at a shear rate of 60 rpm.[11] Biochemical parameters were determined by the use of an auto-analyzer (Tokyo-Boeki Autoanalyzer).

STATISTICAL ANALYSIS

All data were expressed as means ± SD. Students t-test was used for statistical comparison of data. In all cases, the criterion for statistical significance was $p< 0.05$.

The study was approved by the local ethics committe of the Istanbul Univsersity Veterinary Faculty (No 2006/162) and was conducted in accordance with the "Guide for the Care and Use of Laboratory Animals".

RESULTS

Lymphocyte rigidity (k) was found to be significantly increased in cats with NIDDM compared to healthy controls (1.215 ± 0.532 vs. 0.265 ±0.42; $p< 0.01$). In addition, erythrocyte rigidity (b), plasma and whole blood viscosity (230 s^{-1}), total cholesterol, low-density lipoprotein, high-density lipoprotein and glucose levels were significantly higher in the diabetic group (Table 1) (Figures 1-4). Interestingly, there was a positive and significant correlation between lymphocyte deformability and erythrocyte deformability (r= 0.833; $p< 0.001$), plasma viscosity (r= 0.574; $p< 0.01$) and blood viscosity (r= 0.715; $p< 0.001$). However, the correlation between lymphocyte deformability and total cholesterol (r= 0.276), low-density lipoprotein (r= 0.387), high-density lipoprotein (r= 0.193), very low density lipoprotein (r= 0.210), triglyceride (r= -0.109) and glucose levels (r= 0.40) were insignificant ($p> 0.05$ for all). The most remarkable finding on SDS-PAGE was the loss of the band corresponding to a 37 kDA protein in diabetic cats (Figure 5).

TABLE 1: Comparison of various biochemical and hemorheological paramateres between cats with non-insulin dependent diabetes mellitus and healthy controls.

	NIDDM (mean ± SD)	Controls (mean ± SD)	p
Lymphocyte rigidity (κ)	1.215 ± 0.532	0.265 ±0.42	<0.01
Erythrocyte rigidity (β)	29.5 ± 2.74	21.0 ± 1.35	< 0.001
Blood viscosity (230 s^{-1})	4.4 ± 0.2	3.8 ± 0.1	< 0.001
Plasma viscosity (230 s^{-1})	1.4 ± 0.1	1.2 ± 0.1	< 0.01
Total cholesterol (mg/dL)	183.4 ±57.8	106.4±15.6	< 0.001
VLDL (mg/dL)	25.1 ± 10	15.5 ± 7.8	NS
LDL (mg/dL)	24.7 ± 14.6	12.1 ± 5.3	< 0.02
HDL (mg/dL)	133.4 ±44.9	78.7 ± 9.5	< 0.001
Tryglyceride (mg/dL)	56 ± 21	56 ± 37	NS
Glycose (mg/dL)	177.9 ±53.2	99.8 ± 21	< 0.001

NIDDM: non-insulin dependent diabetes mellitus; VLDL: very low density lipoprotein; LDL: low density lipoprotein; HDL: high density lipoprotein; SD: standard deviation; NS: non-significant.

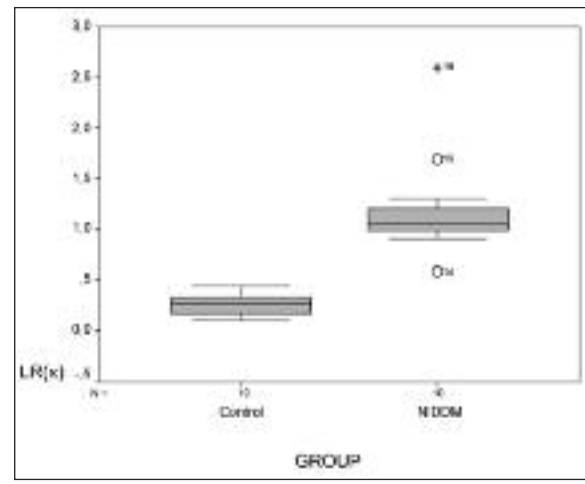

FIGURE 1: Distribution of lymphocyte rigidity values in the diabetic and control groups as shown by box plot graphics. Lymphocyte rigidity is significantly higher in the diabetic group (1.215 ± 0.532 vs. 0.265 ± 0.42; $p< 0.01$). LR, lymphocyte rigidity; NIDDM, non-insulin dependent diabetes mellitus.

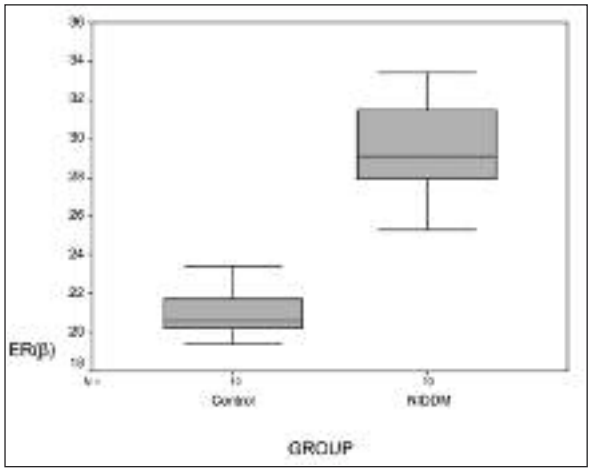

FIGURE 2: Distribution of erythrocyte rigidity values in the diabetic and control groups as shown by box plot graphics. Erythrocyte rigidity is significantly higher in the diabetic group (21.0 ± 1.35 vs. 29.5 ± 2.74; p< 0.001). ER, erythrocyte rigidity; NIDDM, non-insulin dependent diabetes mellitus.

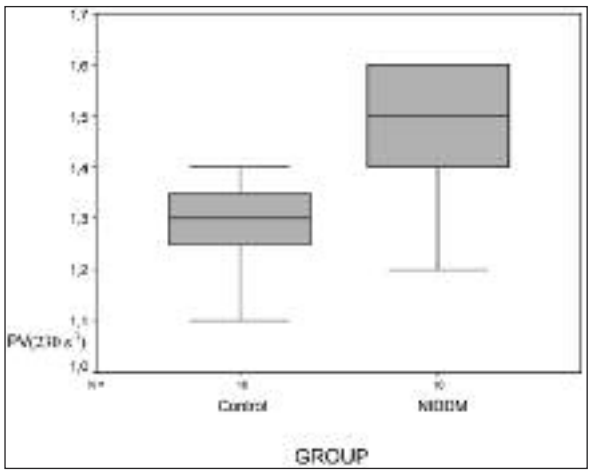

FIGURE 3: Distribution of plasma viscosity values in the diabetic and control groups as shown by box plot graphics. Plasma viscosity is significantly higher in the diabetic group (1.2 ± 0.1 vs. 1.4 ± 0.1; p< 0.01). PV, plasma viscosity; NIDDM, non-insulin dependent diabetes mellitus.

DISCUSSION

Among factors which play a role in the etiopathogenesis of chronic complications (especially diabetic microangiopathy) of diabetes mellitus, hemorheological alterations have been emphasized in various studies. It has been reported that whole blood and plasma viscosity, hematocrit and red blood cell aggregation increase, while red blood cell deformability decreases in diabetes mellitus. Based on the strong association between vascular complications and blood rheology, it was hypothesized that abnormalities in blood rheology may contribute to the development of vascular complications in diabetes mellitus.[3,12-14]

The abovementioned observations can be explained by the biochemical abnormalities which are frequently associated with diabetes mellitus. Especially, abnormalities of plasma lipids (hypercholesterolemia, hypertryglyceridemia) may effect red cell membrane composition and therefore may lead to increased red cell rigidity. It has been reported that fatty acid composition of the erythrocyte

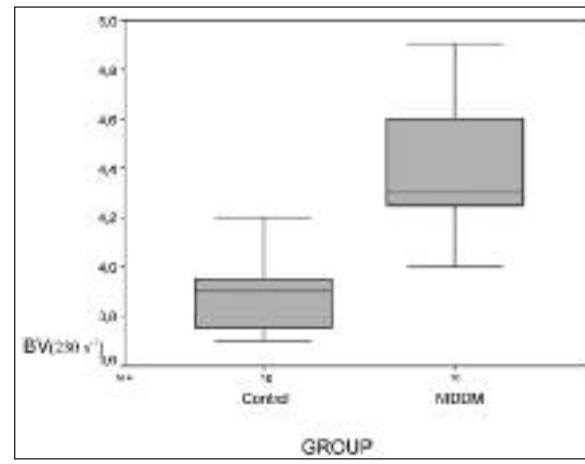

FIGURE 4: Distribution of blood viscosity values in the diabetic and control groups as shown by box plot graphics. Blood viscosity is significantly higher in the diabetic group (3.8 ± 0.1 vs. 4.4 ± 0.2; p< 0.001) BV, blood viscosity; NIDDM, non-insulin dependent diabetes mellitus.

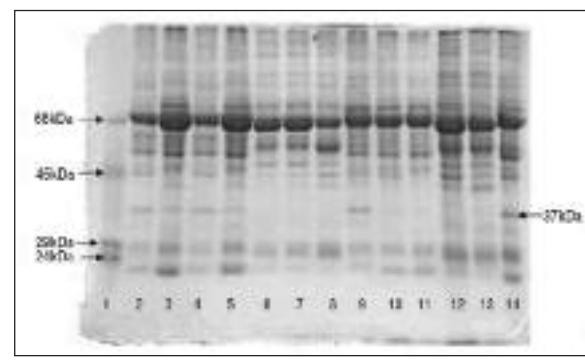

FIGURE 5: SDSP-PAGE pattern of lymphocyte membrane proteins from cats with non-insulin dependent diabetes mellitus (columns 6, 7, 8, 10, 11, 12, 13) and healthy controls (columns 2, 3, 4, 5, 9, 14). A 37 kDa protein is lost in diabetic cats (Pointed by arrow at the right side of the figure). On the first column a reference electrophoresis was run with bovine albumin (66 000 kDa), ovalbumin (45 000 kDa), carbonic anhydrase (29 kDa) and bovine trypsinogene (24 kDa) as standarts. kDA: kiloDalton.

membrane phospholipids is altered in diabetes mellitus.[15-17] Previous studies have also shown an increased eryhrocyte membrane cholesterol level which correlated with erythrocyte deformability.[18,19]

In contrast to erythrocyte deformability, there is relatively limited data concerning leukocyte/lymphocyte deformability and membrane composition in diabetes mellitus. In one of the earliest studies, Ernst and Matrai[20] reported that both red and white blood cell rheology were impaired in type 2 diabetes mellitus. The authors suggested that impaired white cell rheology could contribute to microcirculatory flow abnormalities associated with diabetes.[20] In agreement with this hypothesis, Vermes et al.[14] reported that white cell filterability was reduced in patients with diabetes and it was more marked in patients with retinopathy. Later, Perego et al.[21] reported that white cell filterability (using the St. George filtrometer) was decreased in patients with diabetes, which was most marked in the group of patients with acute vascular events. In an animal study, Masuda et al.[22] showed that polymorphonuclear leukocyte membrane fluidity correlated inversely with blood glucose levels in streptozocine-induced diabetic rats. Kantar et al.[23] assessed polymorphonuclear cell membrane fluidity by steady-state fluorescence anisotropy of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5 hexatriene (TMA-DPH) in patients with insulin dependent diabetes mellitus and concluded that membrane fluidity in resting polymorphnuclear leukocytes (PNL) was decreased in the patient group compared to the controls. Caimi et al.[24] reported that PNL fluidity was decreased in patients with diabetes mellitus and the decrease was more pronounced in patients with type 2 diabetes. In another study conducted by LoPresti et al.[25] it was found that PNL fluidity was different between patients with diabetes mellitus and controls.

Rheological properties of lymphocytes have been even less extensively studied. Athanassiou et al.[26] showed that lymphocytes were more rigid in patients with type II diabetes mellitus using both the filtration and micropipette technique. In a more recent study, Perrault et al.[27] found that lymphocytes from diabetic mice had an equivalent cytoplasmic viscosity to normals by using the micropipette technique in non-obese diabetic rats. However, the higher mean cortical tension (by calculating the recovery time constant, which is derived from the recovery length following expulsion of the lymphocytes from the micropipette) suggested that diabetic lymphocytes are stiffer than control cells.[27]

Although we used a different method (microfiltration technique), we similarly found that lymphocytes were less deformable in cats with diabetes mellitus. The positive correlation between lymphocyte deformability and plasma and whole blood viscosity suggests that increased lymphocyte rigidity may indeed play a role in the disturbed blood flow in the microcirculation. Further, the positive correlation between lymphocyte deformability and erythrocyte deformability implies that the conditions affecting erythrocyte deformability also influence lymphocyte membranes. It is probable that, in a mechanism similar to observed in erythrocytes, lymphocyte membrane composition is altered in diabetes mellitus. Indeed, recently it has been shown that total saturated fatty acid and cholesterol composition were increased in patients with type-2 diabetes mellitus.[28] Whether the same observation applies to fractionated leukocytes remains to be investigated.

In our study, there was no correlation between lymphocyte deformability and blood lipids and glucose levels. Possible explanations include the absence of a direct mechanistic relation between these parameters, small sample size, or the effect of additional mechanisms which are capable of altering lymphocyte membrane more profoundly. In this context, another factor which seems to be ignored so far is the contribution of plasma membrane protein alterations to the increased leukocyte/lymphocyte rigidity. In the present study, we used the SDS-PAGE technique to analyse qualitative/quantitive changes in the protein content of lymphocyte membranes. Interestingly, we observed the loss of a band which corresponded to a 37 kDA protein. The electrophoretic pattern of lymphocyte membrane proteins has not been

described before. Therefore, unfortunately, we were not able to characterize the 37 kDA protein. However, this result implies that diabetes is associated with abnormal protein composition in lymphocyte membranes. This is in agreement with our previous study which focused on erythrocyte membranes in diabetic cats. We have shown that the band corresponding to actin ("band 5" of a standard SDS-P AGE) was weakened and bands 4.5 and 4.9 (corresponding to glycophorin A, B and C integral proteins and dematin, respectively) disappeared.[7]

These findings raise the question of whether the diabetic mileau may effect cell membranes by other means. In this context, a possible effect of increased non-enzymatic glycosylation of membrane proteins in diabetes worths discussine. It is well established that glucose forms early glycosylation products with proteins at a rate proportional to glucose concentration to generate the so-called early glycosylation products. An important example with clinical significance is the glycosylated hemoglobin, or HbA1c.[29,30] Stufano et al.[31] reported that there is a positive correlation between the concentration of glycosylated hemoglobin and erythrocyte rigidity in diabetic patients, although they did not analyze the status of membrane proteins. Indeed, there are few studies demonstrating that glycosylation is not confined to HbA1c and involves erythrocyte membranes.[32-35] Some studies suggested that non-enzymatic glycosylation of the erythrocyte membrane may impair deformability.[36-38] It has also been reported that plasma levels of HbA1c correlate with leukocyte deformability in diabetic patients.[39,40] On the other hand, to our knowledge, neither the glycosylation status of lymphocyte membrane proteins nor their electrophoretic pattern on SDS-PAGE has been investigated before. Therefore, the impact of membrane protein alterations, their relation to glycosylation and influence on blood rheology remain to be studied. In the present study, although we observed an abnormal pattern on SDS-PAGE, we were not able to analyze if this abnormality was associated with glycosylation of membrane proteins. Unfortunately, we were not able to determine blood HbA1c levels, which could help us to show a possible correlation between abnormal glycosylation, abnormalities in blood rheology and altered membrane protein content.

CONCLUSION

To conclude, the results of the present study and previous studies suggest that in NIDDM, decreased deformability is a general phenomenon affecting various types of blood cells including lymphocytes. This is associated with abnormal membrane protein composition, as shown by SDS-PAGE. The mechanism(s) responsible for these abnormalities, such as enzymatic glycosylation of plasma membranes needs to be evaluated by further studies.

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