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Type 2 Diabetes Mellitus Associated with Premature Aging

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Abstract

Background: A decrease of naive T-cells and a concomitant increase in memory cells are the accepted consequences of aging on the adaptive immune system.
Objectives: The current case-control study aimed at considering the impact of chronic hyperglycemia that leads to glycation of modified self proteins on aging via the memory cell percentages among the patients admitted to the endocrinology department of Bulent Ecevit University Hospital in Zonguldak province, Turkey, from September to October 2015.
Methods: Blood samples were collected from 81 patients with diabetes mellitus (DM) and 39 healthy volunteers with no history of autoimmune diseases or chronic inflammatory disorders, based on the purposive sampling method. The patients were divided into 2 groups based on the presence and absence of the diabetic microvascular complications. Diabetic nephropathy, neuropathy, and retinopathy were investigated according to the American Diabetes Association criteria. T-lymphocytes subpopulations were measured in peripheral blood by the flow cytometry. CD27, CD45 RO, and CD45 RA were used to discriminate naive and memory T-cells ($CD4^+$ and $CD8^+$). Data for each subpopulation were reported as a percentage of the total $CD4^+$ and $CD8^+$ cells.
Results: The mean percentage of naive (CD45RA + CD27+) and memory (CD45RO + CD27+ central memory and CD45RO + CD27- effector memory cells) $CD4^+$ T-cells in patients with type 2 DM and healthy controls were 26.73 ± 15.04, 19.21 ± 10.80, 31.35 ± 10.94, and 15.07 ± 6.97, respectively. A decrease in the naive and an increase in memory $CD4^+$ T-cell proportion were found in patients with type 2 DM, compared to the healthy controls (P values = 0.031 and 0.018, respectively).
Conclusions: Higher memory and lower naive $CD4^+$ T-cells probably reflect thr accelerated aging of the adaptive immune system in patients with type 2 diabetes.

Keywords: Diabetes Mellitus, T-Lymphocytes, Memory, Aging

1. Background

Aging is an inevitable process, which is the cumulative impact of disorders created by endogenous and exogenous factors playing a role in aging of the metabolism (1-5). Diabetes mellitus (DM) is a recognized cause of premature aging that may lead to reduction of life expectancy in the patients, compared to the normal population, due to DM-related complications (6, 7). Both aging and DM share 2 important biological processes that damage the body: oxidative stress and glycation (advanced glycosylated end products; AGEs) that play a significant role in the incidence of chronic diseases associated with underlying inflammation (8, 9).

As age advances, the changes of cells in the innate immune system leads to compromised functioning of the immune system. However, the number and frequency of naive T-cells decreases, probably due to the reduction of thymopoiesis, while the frequency and number of memory cells increase with aging. There are several memory subsets in the CD4 and CD8 compartments (10, 11). CD45RA expressing T-cells are initially considered to be naive T-cells that are the precursors of memory T-lymphocytes containing effector and central memory T-cells (12).

2. Objectives

The augmentation of memory T-cells needs a time period as an immunosenescence marker. However, the oxidative stress markers may vary dramatically. Hence, the current study aimed at assessing the relationship between DM and immunosenescence by measuring memory T-cells percentage as a marker of aging by the flow cytometry technique.

3. Methods

3.1. Study Population

In the current case-control study, the data of 112 consecutive patients with type 2 DM and those of 39 healthy volunteers admitted to the Endocrinology Department of Bulent Ecevit University Hospital in Zonguldak province, Turkey, from September to October 2015 were assessed. Using the obtained data, a sample size with a statistical power of 87%, effect size = 0.3, and 0.05 significance level with G-Power 3.0.10 program was determined. The patients were assessed for type 2 DM according to the American Diabetes Association (ADA) criteria (3). Exclusion criteria were the autoimmune diseases and disorders that cause chronic inflammation including sarcoidosis, atherosclerosis, familial dyslipidemia, thyroid dysfunction, infectious diseases, inflammatory bowel diseases, cancers, connective tissue diseases, renal or liver diseases, and smoking. Totally, 7 patients with chronic renal failure (stage 5), 4 patients with rheumatoid arthritis, 11 patients with coronary artery disease, 5 patients with a history of cerebrovascular disease, 3 patients with peripheral arterial disease, and 1 patient with pneumonia were excluded. Finally, 81 patients with type 2 DM and 39 age- and gender-matched healthy volunteers were included in the current study. Screening for diabetic nephropathy or microalbuminuria was accomplished by 24-hour urine collection. Proteinuria > 500 mg and albumin excretion of 30 to 299 mg in 24 hours were defined as diabetic nephropathy and microalbuminuria, respectively. Diabetic retinopathy was diagnosed by retinal examination. The presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes, after the exclusion of other causes, were described as diabetic neuropathy according to ADA criteria (13). The patients were divided into 2 subgroups according to diabetic complications as follows: group A (patients with diabetic microvascular complications; n = 38) and group B (patients with no diabetic complications; n = 43). The controls (group C; n = 39) comprised healthy volunteers. Body mass index (BMI) was calculated as weight of individuals divided by the square of their height (kg/m^2). The characteristics of all participants are shown in Table 1.

3.2. Ethical Considerations

The study protocol conforms to the ethical guidelines of the 1975 Helsinki Declaration, as reflected in a priori approval by Bulent Ecevit University hospital human research committee (the clinical trial registration number: 2015-28-26/05). The aims of the study were explained in detail to the participants. Participation in the study was completely voluntary and free from any obligations to the physician or researchers. The signed informed consent was obtained from all participants.

3.3. Biochemical Analysis

Blood samples were obtained from patients in the morning after at least 8 hours of fasting. The fasting plasma glucose (FPG) was measured by the enzymatic colorimetric method using the glucose oxidase test. Hemoglobin A1c (HbA1c) was analyzed by the latex immunoturbidimetric assay and C-reactive protein (CRP) was measured by Dade Behring BN ProSpec System using a nephelometric method. The equipment used in the current study is calibrated annually. The internal and external controls were applied everyday based on the manufacturers' guidelines.

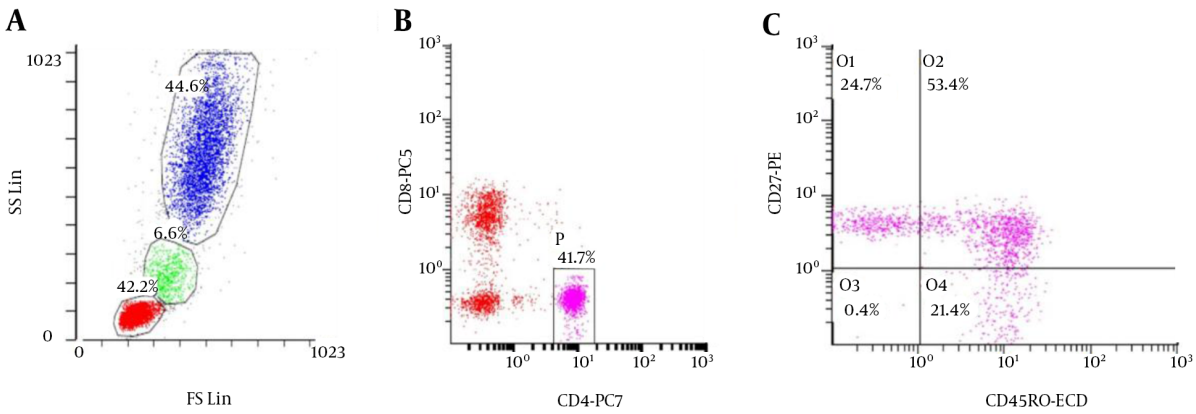
3.4. Complete Blood Count and Flow Cytometric Detection of Memory T-Cells

Automated complete blood count was performed by a Coulter LH780 Hematology Analyzer (Beckman Coulter Inc. Brea., CA, USA). Heparinized blood was used for flow cytometric assessment (Beckman Coulter FC500, USA) to assess T-cell subsets. CD3 and CD19 were used to identify T- and B-cells. CD3$^+$ T-cells were analyzed for CD4 and CD8 cell expression. Then, the study focused on detecting memory T-cells. CD3, CD4, CD8, CD27, CD45RO, and CD45RA were used to discriminate naive and memory T-cells. CD45RA expressing T-cells were initially considered to be naive T-cells. CD3, CD27, and CD45RO triple staining was used to discriminate the total memory T-cell counts. When CD4 gate was selected on CD4, CD27, and CD45RO triple staining, CD27 and CD45RO double positive cells indicated central memory (CD45RO + CD27+) T-cells and only CD45RO positive group indicated effector memory (CD45RO + CD27-) T-cells (Figure 1A - 1C). A similar design was used for CD8$^+$ T-cells. Measurements were obtained by a single observer in the study.

3.5. Statistical Analysis

Statistical analyses were performed by SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Distribution of data was evaluated by the Shapiro-Wilk test. Continuous variables were expressed as mean ± standard deviation (SD) or median (minimum-maximum), and categorical variables as frequency and percentage. Continuous variables were compared by the independent samples t test or the Mann-Whitney U test for the 2 groups. ANOVA or the Kruskal-Wallis test was used to determine the differences between the 3 or more groups. The Tukey test was used as a post hoc test, if ANOVA was statistically significant. The Dunn test was used as a post hoc test after the Kruskal-Wallis test. P

Figure 1. An Example of Gating Procedure for CD4+CD45RO+ Memory Cells



A, Lymphocyte gate (red color); B, $CD4^+$ cells gate (purple); C, CD27 and CD45RO+ cells on CD4 positive T-cells. O2 area indicates central memory cells percentage, O4 area indicates effective memory cells percentage, and O2 + O4 area indicates total $CD4^+$ memory cells. This gating strategy was used for all samples and also other memory T-cells; for instance, $CD8^+$ T-cells.

values < 0.05 were considered statistically significant for all tests.

4. Results

The expression of various surface molecules on T-lymphocytes was examined among the groups (Table 1). No statistically significant difference was observed regarding age (P value = 0.828) and gender (P value = 0.392) between the patients with diabetes and the healthy controls. The CRP values and BMI were higher in the patients with diabetes than the controls (P value = 0.001). Among the patients with diabetes, there were no statistical differences in CRP values, BMI, FPG, and HbA1c, except for the disease duration (P values = 0.443, 0.76, 0.607, 0.194, and 0.003, respectively). The proportions of $CD4^+$ T-cells, $CD8^+$ T-cells, and CD4/CD8 ratio showed no significant differences between the 2 groups (P values= 0.705, 0.568, and 0.179, respectively). A statistically increased percentage of B-cells ($CD19^+$) was observed in the patients with diabetes (10.9% $\pm$ 4.32%), compared to the healthy controls (9.16% $\pm$ 3.73%) (P value = 0.021).

There were no significant differences in the frequencies of CD4+CD45RA+CD27-, CD4+CD45RO+CD27-, CD4+CD45RO+CD27+, CD8+CD45RA+CD27-, CD8+CD45RO+CD27-, and CD8+CD45RO+CD27+ between the groups (P value > 0.05). A decrease in naive and an increase were observed in memory $CD4^+$ T-cell proportions in patients with diabetes, compared to the healthy controls (P values = 0.031 and 0.018, respectively). There was no significant difference between the groups regarding naive and memory $CD8^+$ T-cells (P values = 0.810 and 0.973, respectively).

When the subgroups were compared with each other, regarding T-cell subsets, the higher memory and lower naive $CD4^+$ T-cells were observed in group A (P values = 0.019 and 0.031, respectively). However, no significant differences were detected in the proportion of memory and naive $CD8^+$ T-cells between the 3 groups (P values = 0.998 and 0.555, respectively). The higher memory and lower naive $CD4^+$ T-cell frequencies occurred in group A, compared with the group C (P values = 0.004 and 0.006, respectively); whereas, neither group A-B nor group B-C showed a significant relationship with each other (P value > 0.05), (Table 2).

5. Discussion

The results of the current study indicated that patients with diabetes microvascular complications had higher memory and lower naive $CD4^+$ T-cells compared to the ones with no diabetic complication and the healthy controls, likely suggesting the preferential association of immunological aging with the progressive damage to body in diabetes due to AGEs and glucose-induced inflammation. It is well established that type 2 DM is associated with a systemic inflammatory state (14). Due to AGEs and glucose-induced inflammation, there could be several mechanisms in the pathogenesis of microvascular and macrovascular complications of type 2 DM (15-17). In the current study, however, there seems to be a consistent association between the elevated mean CRP value, BMI, and

Table 1. Baseline Characteristics of the Study Population[a]

Variable	Patients With Diabetes, n = 81	Healthy Controls n = 39	P Value
Mean age, y	55.03 ± 9.95	54.35 ± 10.66	0.828
Gender (male/female)	32/49	13/26	0.392
BMI, kg/m^2	30.94 ± 4.85	27.96 ± 4.36	0.001
CRP, mg/L	8.1 ± 7.7	3 ± 2	0.001
White blood cells, μL	7689.41 ± 1735.07	6871.33 ± 2238.18	0.045
Lymphocytes, μL	2311.76 ± 735.56	2063.15 ± 602.88	0.09
CD19+, %	10.5 (7.95 - 13.15)	8.8 (6.85 - 11.25)	0.021
CD4+, %	46.2 ± 7.6	45.8 ± 7.1	0.705
CD8+, %	26.3 ± 7.4	25.4 ± 8.8	0.568
CD4/CD8	2.01 ± 1.99	3.29 ± 5.23	0.179
CD4+CD45RA+CD27+, %	23.2 (17 - 37.25)	32.1 (23.7 - 38.5)	0.031
CD4+CD45RO+CD27-, %	15.3 (11.95 - 24)	13.5 (10.7 - 19.3)	0.065
CD4+CD45RO+CD27+, %	55.8 (49.9 - 62.1)	52.7 (47.85 - 60.7)	0.421
CD4+CD45RO+, %	76.5 (66 - 76.5)	69.8 (63.4 - 77.3)	0.018
CD8+CD45RA+CD27+, %	29.1 (19.1 - 40.9)	30.2 (20.3 - 40.6)	0.810
CD8+CD45RO+CD27-, %	13.7 (9 - 20.6)	13.5 (8.15 - 20.55)	0.884
CD8+CD45RO+CD27+, %	35.82 ± 11.79	35.06 ± 12.02	0.944
CD8+CD45RO+, %	51.57 ± 13.67	51.41 ± 13.40	0.973

Abbreviations: BMI, body mass index; CRP, C-reactive protein.
[a]Values are expressed as mean ± SD or median (q1 - q3).

DM. Comparing the parameters including CRP, BMI, FPG, and HbA1c, the disease duration emerged as a determining factor reflecting the accumulation of the harmful effects of DM-related pathophysiological changes among the patients with DM.

Pedicino et al. demonstrated that both types of DM were associated with an impaired T-cell balance, characterized by CD4$^+$CD28^{null} T-cell expansion and CD4+CD25+Foxp3+ regulatory T-cell reduction. The expansion of CD4+CD28^{null} T-cells was associated with HbA1c in type 2 DM (18). In another study, Zeng et al. revealed that the levels of CD4+CD25^{hi}Foxp3+ Tregs and CD4+CD25^{hi} CD127- Tregs decreased in the periphery of patients with type 2 DM, whereas the proinflammatory Th17 and Th1 cells increased, indicating that distinct CD4$^+$ T-cell subset polarization occurred in patients with type 2 DM (19). It is well demonstrated that CD4+CD25hi Tregs had the ability to downregulate the function of innate and adaptive immune effector cells. Thus, dysfunction of CD4+CD25^{hi} Tregs promotes the development of autoimmune diseases in human beings (20). However, the mechanisms are not well defined. The results of previous studies suggested that the development of unusual T-cell subsets might be influenced by the persistent poor glycemic control and might be presumably related to the presence of high levels of AGEs.

Based on the alterations of T-cell subsets, a marked decrease in naive and also an increase in memory CD4$^+$ T-cell proportions were noticed in the patients with type 2 DM, compared to the healthy controls; whereas the subsets of CD8$^+$ T-cells showed no significant difference between the 2 groups. Furthermore, there were no significant differences between the groups regarding CD4$^+$ and CD8$^+$ T-cells, and CD4/CD8 ratio. There are a limited number of studies on the chronic adaptive immune activation during the pathogenesis of type 2 DM. In non-insulin-dependent diabetes mellitus (NIDDM) subjects, a report that analyzed the T-cell subsets after phytohemagglutinin stimulation by flow cytometry indicated no statistically significant differences between the subsets of CD4$^+$ and CD8$^+$ T-cells from peripheral blood of patients with diabetes and the healthy controls (21). Olson et al. investigated whether high degree of chronic adaptive immune activation, characterized by higher memory and lower naive CD4$^+$ T-cells, was positively associated with type 2 DM (22). It was revealed that the associations of higher memory and lower naive CD4$^+$ T-cells with type 2 DM was consistent with the role of chronic adaptive immune activation. They recommended that the activation of CD4$^+$ memory cells that recognize non-pancreatic beta-cell autoantigens such as oxidized low-density lipoprotein (LDL) or intestinal-derived products by a proinflammatory cytokine environment generated during obesity or type 2 DM may also account for such relationships. However, the relationships of higher memory and lower naive CD4$^+$ T-cells with type 2 DM may reflect both direct and/or indirect mechanisms triggered by diabetogenic, obesity-associated antigens, and pathogens with cross-reactivity to beta-cell antigens that may have implications for T-cell activation in type 2 DM (23-25).

T-cell subsets associated with premature immune aging are also assessed among patients with end-stage renal disease (ESRD). Betjes et al. reported that younger patients (25 to 45 years old) with ESRD resembled older healthy controls (60 to 80 years old), as they had a significant loss of naive T-cells and a relative increase in memory T-cells showing progressive terminal differentiation (26). In accordance with the previous studies, Meijers et al. revealed that uremia caused premature immune aging of the T-cell

Table 2. Characteristics of the Study Groups and Proportion of Peripheral Lymphocyte Subsets[a]

T Subsets	Group A (n = 38)	Group B (n = 43)	Group C (n = 39)	P Value
Mean age, y	56.51 ± 11.48	53.65 ± 8.17	54.35 ± 10.66	0.441
Gender (male/female)	13/25	19/24	13/26	0.478
BMI, kg/m^2	31.7 ± 4.5	30.1 ± 5	27.96 ± 4.36	0.001
CRP, mg/L	8.4 ± 10.2	7.8 ± 3.4	3 ± 2	0.001
Disease duration, mo	114.9 ± 86	62.3 ± 63.3	-	0.003
FPG, mg/dL	184.8 ± 95.3	165.7 ± 57.5	99.1 ± 8.2	0.001
HbA1C, %	8 ± 2.1	7.6 ± 2.1	-	0.194
CD4+CD45RA+CD27+, %	21.05 (14.1 - 34.4)	26.7 (18.5 - 38.1)	32.1 (23.7 - 38.5)	0.031
CD4+CD45RO+, %	77.45 ± 11.83	72.80 ± 14.12	69.31 ± 11.09	0.019
CD8+CD45RA+CD27+, %	26.4 (17.5 - 34.6)	31.6 (21.4 - 44)	30.2 (20.3 - 40.6)	0.555
CD8+CD45RO+, %	51.55 ± 12.93	51.58 ± 14.44	51.41 ± 13.40	0.998

Abbreviations: BMI, body mass index; CRP, C-reactive protein; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c.
[a]Values are expressed as mean ± SD or median (q1 - q3).

system in the patients group, compared to the healthy controls (27).

The effects of aging on the immune system are widespread. It is proposed that the ratio of naive T-cells to memory T-cells declines, an increase in the number of memory T-cells is now a particularly well-recognized feature of aging (10, 28-31). Based on the current study findings, an increase of memory CD4$^+$ T-cells was observed in the patients with type 2 DM and controls with aging; while, naive CD4$^+$ T-cells appeared not to be associated with age in both groups. Such alterations can be a potential explanation for age-dependent changes in subsets of CD4$^+$ T-cells. Consistent with the previous reports (32-35), the naive CD8$^+$ T-cells were inversely correlated with age, and there was no relationship between memory CD8$^+$ T-cells and age in both groups. Age-associated alterations including diminished thymopoiesis and the effects of chronic antigenic stimulation may provide an explanation for the proportion of CD8$^+$ T-cell subsets.

5.1. Strengths and Limitations

The strong point of the current study was its clear definition for DM-related immunosenecence using flow cytometry. The main limitation of the current study was the enrolment of a relatively small number of patients from a single center, which could lead to selection bias. To minimize the selection bias, it was tried to include all patients who met the inclusion criteria. Nevertheless, future comprehensive studies covering larger populations to clarify accelerated aging in patients with type 2 DM are recommended.

5.2. Conclusions

Due to AGEs and glucose-induced inflammation, type 2 DM might affect the premature aging of the adaptive immune system. Higher memory and lower naive CD4$^+$ T-cells can probably be an eligible parameter to reflect accelerated aging during the progression of type 2 diabetes. The appropriate therapy for DM would be a good choice that ensures a reduction in the risk of DM-related complications and accelerated aging in patients with type 2 DM.

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Footnotes

Authors' Contribution: Study concepts: Ishak Ozel Tekin and Muammer Bilici; study design: Dilek Karakaya Arpaci and Muammer Bilici; study supervision: Ishak Ozel Tekin; literature search: Sevil Uygun Ilikhan, Muammer Bilici, and Basak Delikanli Corakci; data collection and processing: Muammer Bilici and Dilek Karakaya Arpaci; analysis and interpretation of data: Mehmet Arasli, Muammer Bilici, and Ishak Ozel Tekin; writing of the manuscript: Muammer Bilici and Basak Delikanli Corakci; critical reviews: Taner Bayraktaroglu and Ishak Ozel Tekin.

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