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CASPASE LEVELS IN THE EVALUATION OF APOPTOSIS IN VITILIGO PATIENTS

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ABSTRACT:

Purpose: Vitiligo is a common cutaneous disorder that is characterized by depigmentation of skin and mucosae due to the loss of melanocytes. The etiology of melanocyte destruction in vitiligo remains largely unknown. Apoptosis, or programmed cell death, is one way of cell destruction. In this study, we aimed to analyze the apoptotic peripheral blood mononuclear cells (PBMCs) and the serum caspase-8 and -9 levels of patients and compare these levels with those of healthy patients in order to determine the possible role of apoptosis in etiopathogenesis.

Materials and Methods: In this study, 33 vitiligo patients and 26 age- and sex- matched control subjects were included. The analyses of PMNCs (lymphocytes) were performed under a fluorescent microscope and viable and apoptotic cells were calculated. Apoptotic index is defined as the ratio of the number of the apoptotic cells to the total cell number. Serum caspase-8 and caspase-9 levels were measured by enzyme-linked immunosorbent assay.

Results: Mean apoptotic index of patients (9.88±1.79) was significantly higher than that of the controls (5.18±1.52) ($p<0.001$). Serum caspase-8 and caspase-9 levels did not differ significantly between patients and controls. Caspase-9 levels were inversely correlated with disease duration ($p= 0.019$).

Conclusion: An increase in apoptosis in the peripheral blood mononuclear cells may play a role in vitiligo pathogenesis.

Key words: Vitiligo, Caspase-8, Caspase-9, Apoptosis

VİTİLİGO HASTALARINDA APOPTOZİSİN DEĞERLENDİRİLMESİNDE KASPAZ DÜZEYLERİ

ÖZ:

Amaç: Vitiligo melanosit kaybı nedeniyle cilt ve mukozanın depigmentasyonu ile karakterize yaygın kutanöz bir hastalıktır. Vitiligoda melanosit yıkımının etyolojisi bilinmemektedir. Programlı hücre ölümü olan apoptozis hücre yıkımı yollarından biridir. Bu çalışmada, etyopatogenezde apoptozisin muhtemel rolünü araştırmak için hastaların apoptotik periferal mononükleer hücrelerini ve serum kaspaz-8 ve 9 düzeylerini analiz etmeyi ve bu düzeyleri sağlıklı kişilerinki ile kıyaslamayı amaçladık.

Gereç ve Yöntemler: Çalışmamıza 33 vitiligo hastası ve 26 yaş ve cinsiyet uyumlu sağlıklı kontrol kişileri dahil edildi. Periferal mononükleer hücrelerin analizi florasan mikroskop altında yapıldı ve apoptotic hücreler belirlendi. Apoptotik indeks apoptotik hücrelerin total hücreye oranı olarak tanımlandı. Serum kaspaz-8 ve kaspaz-9 düzeyleri Enzyme-linked Immunosorbent Assay yöntemi ile analiz edildi.

Bulgular: Hastaların ortalama apoptotik indeksi (9.88±1.79) sağlıklı kontrollerinkinden (5.18±1.52) anlamlı derecede yüksekti ($p<0.001$). Serum kaspaz-8 ve kaspaz-9 düzeylerine bakıldığında hasta ve kontrol grubu arasında anlamlı bir fark yoktu. Kaspaz-9 düzeyleri ile hastalığın süresi arasında negatif korelasyon vardı ($p= 0.019$).

Sonuç: Bu bulgulara göre, periferal mononükleer hücrelerin apoptozisindeki artış vitiligonun patogenezinde rol oynuyor olabilir.

Anahtar kelimeler: Vitiligo, Kaspaz-8, Kaspaz-9, Apoptozis

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INTRODUCTION

Vitiligo is a pigmentary disorder characterized by melanocyte loss in the involved skin. Although its etiopathogenesis has not been clarified yet, environmental, immunological, and genetic hypotheses have been suggested[1]. Since it is not known where the pathologic events start first, various studies with special focus on different offender cells, melanocytes, keratinocytes, and peripheral blood mononuclear cells (PBMCs) have been conducted[2-5]. Studies investigating PBMCs in vitiligo patients illuminated different aspects of the changes in these cells and contributed to the knowledge about the relationship between periphery and skin[4-6]. Apoptosis either in melanocytes or keratinocytes has been studied by many authors as a part of immunologic events in the development of vitiligo[2,3,7]. Apoptosis is the process of programmed cell death and it can be initiated by either extracellular or intracellular signals, which result in the activation of extrinsic or intrinsic pathway of death, respectively. Caspases are serine proteases involved in the processing of apoptosis. Caspase-8 plays a role in the extrinsic pathway, whereas caspase-9 plays a role in the intrinsic pathway[8,9]. In the present study, we aimed to examine whether PBMCs of untreated vitiligo patients differ from those of healthy subjects in terms of apoptosis. We also analyzed the serum caspase-8 and -9 levels of patients and compared these levels with those of healthy patients in order to determine the possible role of these proteases in etiopathogenesis.

MATERIALS AND METHODS

Patients and controls

Thirty-three vitiligo patients with nonsegmental vitiligo and 26 age- and sex-matched control subjects were included in the study. All the enrollees gave informed consent and the study protocol was approved by the ethics committee of our hospital. The exclusion criteria were having a history of systemic illnesses such as diabetes mellitus, renal and hepatic insufficiency, internal malignancies, using any systemic drugs, and being a smoker.

Blood collection

None of the patients had used any systemic agent for the treatment of the disease previously. Patients were instructed not to use any topicals 2 weeks prior to the blood sampling. Blood samples (10 mL) were collected after a rest of 30 minutes at 9:00 a.m. following an overnight fast, in sitting position, by a 25-gauge needle through antecubital vein, avoiding hemolysis.

Laboratory methods

Measurement of serum caspase-8 and caspase-9 levels

Serum was obtained by the centrifugation of the collected blood and immediately stored at -80 °C until use. Serum caspase-8

levels were measured by enzyme-linked immunosorbent assay (ELISA) using commercial kits (Bender MedSystems, Vienna, Austria). The minimum detectable concentration for caspase-8 was 0.10 ng/mL. Intra-assay and inter-assay variation coefficients for caspase-8 were <6.7% and <8.5%, respectively[10].

Serum caspase-9 levels were also measured by ELISA using commercial kits (Bender MedSystems, Vienna, Austria). The minimum detectable concentration for caspase-9 was 0.40 ng/mL. Intra-assay and inter-assay variation coefficients for caspase-9 were <6.6% and <9.0%, respectively[11,12].

Table 1: Results of the mean apoptotic index, serum caspase-8, and serum caspase-9

	Vitiligo patients	Control group	P
Mean apoptotic index of the of PBMCs	9.88±1.79	5.18±1.52	<0.001
Serum caspase-8 (ng/ml)	0.19±0.03	0.19±0.02	0.330
Serum caspase-9 (ng/ml)	1.84±0.33	1.95±0.40	0.236

Lymphocyte isolation and morphological assessment of apoptosis

Lymphocyte isolation

Peripheral venous blood was drawn from patients and controls into heparinized Vacutainer™ tubes. Blood samples were layered on Ficoll and centrifuged at 500 g for 10 min to separate mononuclear cells. The buffy coat was recovered and washed twice with RPMI 1640 (Biological Industries).

Morphological assessment of apoptosis

After isolation of the lymphocytes, the cell pellets were collected on a glass slide, stained with 1 µL of a mixture of acridine orange (Sigma A-6014, 100 µg/mL) and ethidium bromide (100 µg/mL, Sigma E-8751) in PBS, and immediately examined under a fluorescence microscope at a 490 nm excitation wavelength. Acridine orange, a vital dye, enters cells through an intact cytoplasmic membrane and intercalates into DNA making it appear green, with structure variations in fluorescence intensity in normal nuclei due to the relative distribution of euchromatin and heterochromatin. In contrast, apoptotic nuclei have condensed chromatin, which is uniformly stained, and takes the form of crescent-shaped or numerous featureless bright spherical bodies. Passive diffusion of acridine orange induces, in addition, a green cytoplasmic coloration. Ethidium bromide is only taken up by cells with a damaged cytoplasmic membrane and stains the nucleus red, with the same characteristic apoptotic features in the case of secondary necrosis or intact nuclear structure in cell death due to primary necrosis[13,14].

Analysis of the lymphocytes

The analyses were performed under a fluorescent microscope. Viable and apoptotic cells were calculated. Apoptotic index is defined as the ratio of the number of the apoptotic cells to the total cell number.

Statistical analysis

Statistical analyses were carried out with SPSS for Windows version 15.0 (SPSS Inc., Chicago, IL, USA). Continuous variables are presented as mean ± standard deviation and categorical variables as percentages. Continuous variables were examined for normality by Shapiro-Wilks test. For normally distributed variables, differences between the groups were determined by t test. A Mann-Whitney test was used for variables not normally distributed. Associations between the continuous variables were investigated by Pearson correlation coefficient or Spearman rank correlation coefficient. Wilcoxon's signed rank test was used to examine the difference between before and after the treatment. The level of significance was 0.05.

RESULTS

Demographic features such as the mean age and gender distribution of vitiligo patients (18 male, 15 female; mean age 34.0±13.7 years) and control subjects (15 male, 11 female; mean age 37.9±10.6 years) were similar. The mean duration of disease was 67.3±63.8 months (median 36 months).

The mean apoptotic index of patients (9.88±1.79) was significantly higher than that of the controls (5.18±1.52) ($p<0.001$). Mean serum caspase-8 levels were 0.19±0.03 ng/ml and 0.19±0.02 ng/ml for patients and controls, respectively. Mean serum caspase-9 levels were 1.84±0.33 ng/ml and 1.95±0.40 ng/ml for patients and controls, respectively. As caspase-8 and caspase-9 levels did not differ significantly between patients and controls (p values 0.330 and 0.236, respectively), their levels did not significantly correlate with the apoptotic index of their corresponding group. Caspase-9 levels were inversely correlated with disease duration ($p=0.019$). Caspase-8 and -9 levels were not correlated significantly in either patients or controls.

DISCUSSION

Although several theories including autoimmune, genetic, and excessive reactive oxygen species have been suggested for the development of vitiligo, no convincing mechanism has been proved (15). It was demonstrated that alteration of the antioxidative pathway in PBMCs of vitiligo patients resulted in overproduction of reactive oxygen species (ROS) and this was associated with the apoptosis of these cells (5). In a subsequent study, these authors suggested that a defect in the mitochondria of PBMCs of vitiligo patients results in the opening of permeability transition pores (PTPs) and then apoptosis through the activation of caspase-3 (4). Another study exploring the connection between the periphery and skin found DNA strand breaks to be elevated in peripheral blood cells of active vitiligo patients (6). It is well known that once the

DNA damage signal is initiated in the cell, it results in the apoptosis of cell by many different operating pathways (16). In our study, the mean apoptotic index of vitiligo patients was significantly higher than that of the controls, which is a result in concordance with previous studies (4-6). By analyzing serum caspase-8 and -9 levels, we also investigated whether apoptosis is related to the intrinsic or extrinsic pathway. Since caspase-8 and caspase-9 levels did not differ significantly between patients and healthy subjects, it is unlikely that these caspases have a central role in the apoptotic death of PBMCs in patients. It is possible that in our patients an apoptotic pathway independent of caspase was involved (9, 16). However, although caspase-9 is not primordially used, it may still have a role in pathogenesis. Since caspase-9 levels are significantly lower in patients with longer disease duration, it can be suggested that its level decreases as the initial inflammatory process subsides. Although we have little data to comment on, we can conclude that this may be a secondary result of the events related to the apoptosis of PBMCs. Further studies investigating the immunologic mechanisms that result in the apoptosis of PBMCs will add to the data about vitiligo pathogenesis.

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