The Relationship Between Apoptosis and Clinical Stages in Sarcoidosis

Sarkoidozda Apoptozisin Klinik Evreler ile İlişkisi

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ABSTRACT Objective: Sarcoidosis is a chronic inflammatory disease of unknown etiology characterized by the formation of non-necrotizing granulomas. The prognosis of this disease varies considerably. The mechanisms leading to the persistence of granuloma formation and lung fibrosis in some of the patients have not been fully established. Apoptosis has been considered as an important process because it limits the inflammation and tissue injury, and promotes the resolution of inflammation. In this study, the role of apoptosis on the progression and resolution in sarcoidosis was investigated. **Material and Methods:** Forty three patients with newly diagnosed pulmonary sarcoidosis were included in this study. The presence and distribution of apoptotic cell death were quantitatively analyzed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) procedure on lung and lymph node tissue sections of biopsy specimens of patients with sarcoidosis and the correlation between the results of TUNEL number and the stage of the patients were analysed. **Results:** Based on the chest radiographs, the patients were classified as stage I (n=20), stage II (n=16), stage III (n=4), or stage IV (n=3). Significant correlation between the number of TUNEL positive cells/1mm^2 and the clinical stages were detected. While the mean number of TUNEL-positive cells in low stage diseases (stage I-II) was 9.69, in high stage diseases (stage III-IV), the mean number was 2.71. **Conclusion:** These findings suggest that apoptosis may be associated with the course of the granulomatous inflammation in sarcoidosis. Therefore, modulation of apoptosis may be a novel strategy to the treatment of sarcoidosis.

Key Words: In situ nick-end labeling; apoptosis; granuloma; sarcoidosis

ÖZET Amaç: Sarkoidoz, etyolojisi bilinmeyen, non-nekrotizan granülom oluşumları ile karakterize kronik inflamatuar bir hastalıktır. Sarkoidozun klinik gidiş ve prognozu farklılıklar göstermektedir. Bir kısım hastada izlenen, granülomatöz inflamasyonun devamlılığına ve akciğer fibrozisine yol açan mekanizma hala tam olarak anlaşılamamıştır. Apoptozisin, sarkoidozda izlenen inflamasyon ve doku hasarının sınırlanması ve dolayısıyla da hastalığın prognozunda etkisi olduğu düşünülmektedir. Bu çalışmada, apoptozisin sarkoidoz gelişimi ve iyileşmesi üzerindeki etkisi araştırılmıştır. **Gereç ve Yöntemler:** Pulmoner sarkoidoz tanısı almış toplam 43 olgu çalışmaya alındı. Hastalara ait akciğer ve lenf nodu doku kesitlerinden TUNEL (terminal deoxynucleotidyltransferase (Tdt)-mediateddeoxyuridinetriphosphate(dUTP)-biotinnickend-labelling), metodu ile in situ DNA fragmanlarının saptanması ile apoptotik indeks belirlendi ve TUNEL sonuçları ile hastaların evreleri arasında ilişki olup olmadığı araştırıldı. **Bulgular:** Radyolojik bulgulara göre değerlendirilen 43 hastanın 20 (%46,5)'si evre I, 16 (%37,2)'sı evre II, 4 (%9,3)'ü evre III ve 3 hasta da (%7) evre IV idi. Bir mm^2'deki TUNEL pozitif hücre sayısı ile hastaların klinik evreleri arasında belirgin ilişki saptandı. Düşük evreli (Evre I-II) hastalarda ortalama TUNEL pozitif hücre sayısı 9,69 iken, ileri evre (Evre III-IV) hastalarda 2,71 idi. **Sonuç:** Bulgular, apoptozisin sarkoidozda izlenen granülomatöz inflamasyonun iyileşmesi ve dolayısıyla hastaların prognozunda etkili olabileceğini desteklemektedir. Bu da sarkoidoz tedavisinde kullanılabilecek ve apoptozis düzenlenmesi üzerine etkili olabilen yeni tedavi seçeneklerini akla getirmektedir.

Anahtar Kelimeler: İn situ çentik uç işaretleme; apoptoz; granülom; sarkoidoz

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Sarcoidosis is a multi-organ disease of unknown etiology, which frequently affects the lungs but also commonly involves the lymph nodes, liver, spleen, skin, heart, eye and other organs. The diagnosis is established when clinical and radiological findings are supported by histologic evidence of non-caseating granulomatous inflammation. The granuloma consists of highly differentiated mononuclear phagocytes (epithelioid cells and multinucleated giant cells) surrounded by lymphocytes.[1] The disease either resolves spontaneously or develops into a more chronic disease where the sarcoid granulomas develop fibrotic changes, which in the airways may lead to a progressive loss of lung function. Factors that influence granuloma formation and the development of fibrosis are not well understood in sarcoidosis.[2-4]

Although the aetiology of sarcoidosis remains unknown, there is no doubt that the pathogenesis of this disease is influenced by various immunological alterations, including depression of cutaneous delayed-type hypersensitivity and an activation of monocytes/macrophages and a heightened helper T cell type I immune response at sites of disease but the mechanism leading to the persistent accumulation of inflammatory cells is not well understood.[5]

Apoptosis, or programmed cell death, is a physiological, genetically controlled, cellular response to external and internal stimuli whose purpose is to eliminate unwanted cells, including infected cells, while preventing damage to surrounding cells or tissue. Several studies suggest that a number of apoptosis-regulatory molecules control immune mechanisms involved in granuloma formation. To date, there have been few studies concerning the alterations of apoptosis in sarcoidosis and the results have been controversial.[4-13] In this study, we evaluated the apoptotic cell counts in the course of sarcoidosis.

MATERIAL AND METHODS

This study, which was included 43 patients with diagnosed sarcoidosis in the Departments of Pathology and Pulmonary Diseases, Gazi University, between 2008 and 2011, was approved by local Ethics Committee of the University. All patients had a typical clinical and radiographic picture compatible with the diagnosis in addition to an elevated CD4/CD8 T cell ratio in the bronchoalveolar lavages (BAL) and/or a biopsy showing non-necrotizing granulomas. The diagnosis of sarcoidosis was made according to the guidelines of the American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders statement on sarcoidosis.[14] The final diagnosis of sarcoidosis based on histological, clinical and radiological evidences. The diagnosis was histologically confirmed by transbronchial lung biopsies in 30 patients (Figure 1a)

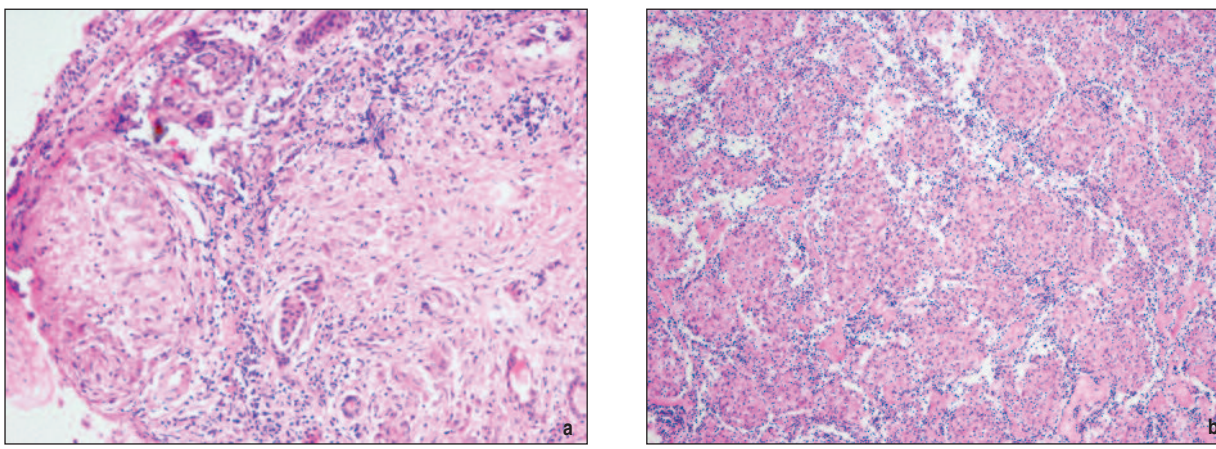

FIGURE 1: a) Well-formed non-necrotizing granulomas, localized in bronchiolar submucosa and **b)** mediastinal lymph node. (H&E,x100).
(See color figure at http://www.turkiyeklinikleri.com/journal/akciger-arsivi/1309-0119/)

and by mediastinal lymph node biopsies in 13 patients (Figure 1b). Ten non-smoking healthy volunteers with normal chest radiographs were included as healthy controls.

Clinical and relevant laboratory findings, including erythrocyte sedimentation rates, serum angiotensin-converting enzyme (ACE) levels, serum C reactive protein (CRP) levels and, pulmonary function tests (PFTs) were reviewed. The patients were staged according to the findings on their chest radiographs.

To evaluate the quantity of apoptosis, biopsy specimens of patients - diagnosed according to the above-defined criteria -showing non-necrotizing epithelioid cell granuloma formation compatible with sarcoidosis were gathered. TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end-labelling) staining was performed to detect *in situ* DNA fragmentation as a marker of apoptosis using an In Situ Apoptosis Detection Kit (Takara Bio, Otsu, Japan), according to the protocols described by Gavrieli; sections were deparaffinized and dehydrated, and endogeneous peroxidase activity was blocked in 0.3% hydrogen peroxidase in PBS for 30 minutes.[15] Sections were incubated at room temperature for 10 minute with equilibration buffer, followed by 1 hour incubation at 37°C with TdT enzyme (or reaction buffer negative controls) diluted with the reaction buffer in a humidity chamber. The TdT reaction was stopped with stop/wash buffer, and sections were washed with PBS before 30-minute incubation with antidigoxigenin conjugated with HRP. Following washing, TUNEL-positive color development was obtained by incubating the section with 0.05% 3,3-diaminobenzidinetetrahydrochloride. Slides were counterstained with heamatoxylen. A positive reaction for apoptosis was characterized by brown/black coloration of the nuclear or perinuclear region of the cell.[15] TUNEL positive cells were counted in histiocytes in the epitheloid granulomas in lung and lymph node tissues according to the number of nuclei labelled in 1 mm^2.

The statistical analysis was performed using SPSS 16.0 software. All values were presented as mean ±SEM (Standart Errors of Mean) and were analyzed with Kruskal-Wallis test followed by the Mann-Whitney U test. Value of p below 0.05 was considered statistically significant.

RESULTS

The clinical and radiological features and laboratory parameters were demonstrated (Table 1). Forty three patients (31 females and 12 males with a median age of 53 years, ranging from 26 to 75) with newly diagnosed pulmonary sarcoidosis were evaluated. The mean serum level of ACE, CRP and ESR were 69.67±31.49, 10.97±10.42, 25.18±17.52 respectively.

Considering the chest radiographs, 20 patients (46.5 %) showed bilateral hilar lymphadenopathy without parenchymal infiltrates and were classified as stage I. Sixteen patients (37.2%) had both hilar lymphadenopathy and parenchymal infiltrates (stage II).

TABLE 1: Clinical, radiological and laboratory characteristics of patients with pulmonary sarcoidosis.

Characteristics	Patients N=43
Age (years)	49.72±11.25
Female/Male	31/12
Sedimentation Rate	25.18±17.52
CRP	10.97±10.42
ACE (µg/L)	69.67±31.49
FEV_1 %	82±23
FVC%	84±24
FEV_1/FVC	79±9
DLCO %	88±25
DLCO VA%	104±20
Chest radiographic stages	
Stage I	20 (46.5%)
Stage II	16 (37.2%)
Stage III	4 (9.3%)
Stage IV	3 (7%)

CRP: C reactive protein; ACE: Angiotensin-converting enzyme; FEV_1: forced expiratory volume in one second; FVC: Forced vital capacity; DLCO: Diffusing Capacity of the Lung for Carbon Monoxide; DLCO VA: diffusing capacity of the lung for carbon monoxide divided by alveolar volume. Results were expressed as mean±SEM.

Four patients (9.3%) presented with parenchymal infiltrates without lymphadenopathy (stage III) and three patients (7%) had pulmonary fibrosis and was considered as stage IV.

For the statistical analysis, the patients were re-grouped based on the radiographic stage: stages I and II with self-limiting clinical course (low stage disease), and the stages III and IV with high probability of disease progression (high stage disease).

The mean number of TUNEL-positive cells was compared among patients grouped according to their radiological stage (Table 2).

The mean number of TUNEL-positive cells/mm^2 was 8.55±5.89, ranging between 2 to 24 (Figure 2, 3). The mean number of TUNEL-positive cells/mm^2 in sarcoid granulomas was higher in low stage disease (9.69 ±5.77, stage I-II) than in patients at high stage disease (2.71±1.25, stage III-IV). Although this study contained a relatively few numbers of patients with high stage disease; there was a statistical significance between apoptotic index detected by TUNEL and patient's stage (p=0.001). However, there was no association with other clinical features. Additionally, no significant relationship was found between stage and clinical and laboratory parameters.

DISCUSSION

It is now well established that sarcoidosis is a granulomatous disorder resulting from an uncontrolled cell-mediated immune reaction in response to unknown antigens. This reaction is characterized by the presence of typical granulomas at the sites of the disease, made of activated macrophages (epithelioid cells) and T lymphocytes (especially CD 4$^+$ T cells). The interactions between both cell types lead to the production of numerous inflammatory mediators, such as IL-2, IFN-γ and tumor necrosis factor (TNF-α), which are essential for the development of granulomas. The benefit of granuloma formation for the host is that it isolates the inflammation, protects the surrounding healthy tissue, controls the growth of pathogens, and prevents systemic dissemination.[1-3]

Although some patients with sarcoidosis show a self-limited clinical course, scattered granulomas

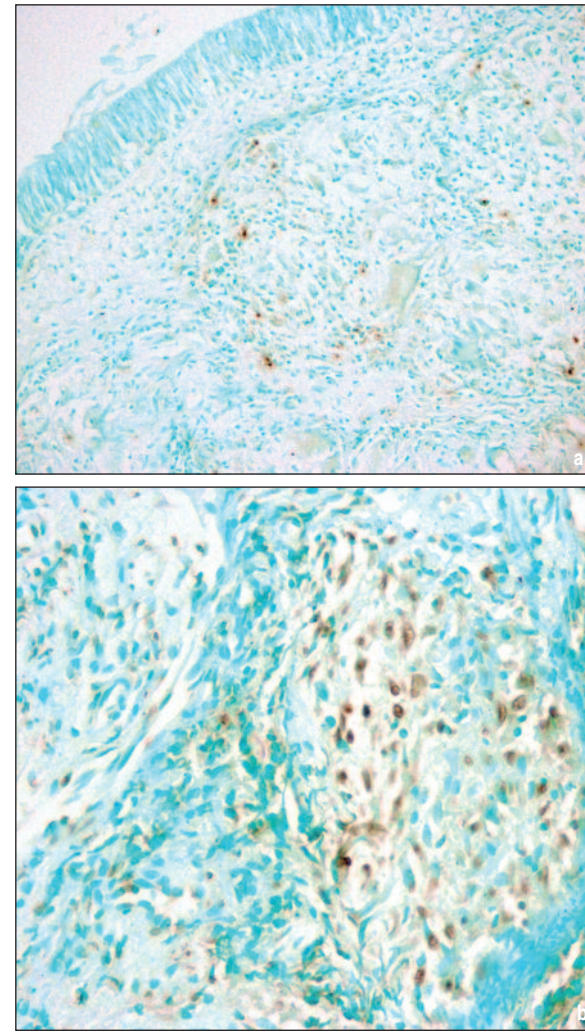

FIGURE 2: Apoptosis in sarcoidosis by the TUNEL assay. **a, b)**TUNEL-positive cells were seen within granulomas in bronchiol mucosa with low stage sarcoidosis (TUNEL, x 100, TUNEL, x400)
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TABLE 2: Correlations between the radiological stage and TUNEL-positive cells in patients with pulmonary sarcoidosis.

		TUNEL-positive cells/mm^2		
Stage	Number of patients	Mean	Minimum	Maximum
I	20	10.10	2	24
II	16	9.18	2	18
III	4	2.75	2	5
IV	3	2.66	2	4
Total	43	8.55	2	24

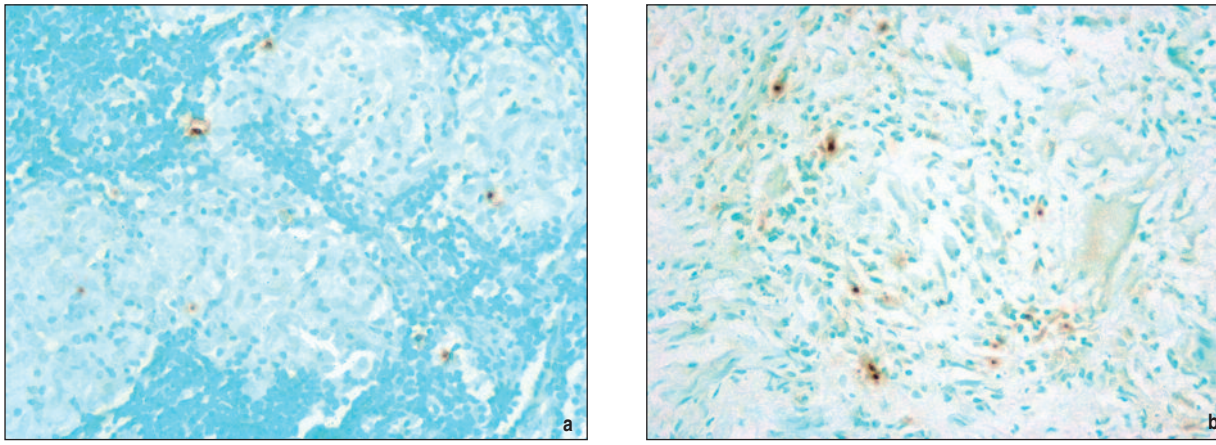

FIGURE 3: a,b) TUNEL-positive cells were seen in histiocytes and lymphocytes within granulomas in lymph node with high stage sarcoidosis (TUNEL, x400)
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within the involved tissues, and spontaneous resolution, other patients show a persistent inflammation and massive development of granulomas that do not recover even if a strong immunosuppressive therapy is used. The mechanisms leading to the persistence of granuloma formation and lung fibrosis have not been fully established. It is thought that, apoptosis plays a crucial role in resolution of inflammation.[4-11]

Apoptosis is a physiological, genetically regulated cell death mechanism that is essential for normal biological processes, including regulation of immune responses. Activated immune cells can be eliminated by apoptosis and thereby their destructive potential may be limited. Both positive and negative regulation of apoptosis of cells influence the initiation, progression, and healing of tissue damage and the balance between these two regulations may critically influence the inflammatory process.[15-19]

To date, only a small number of experimental and clinical studies on alternations of apoptosis in sarcoidosis have been reported and the results have been controversial.[4-13]

The first study about the role of apoptosis in sarcoidosis was evaluated by Cree and co-workers.[6] Apoptotic bodies were counted by scanning the selected area at a magnification of x400, by light microscopy. They suggested that apoptosis play a major part in the regression of granulomata and thus may have an important role in disease outcome.

Agostini et al reviewed that, a number of molecules involved in the regulation of apoptosis had been evaluated in sarcoidosis, including members belonging to the superfamilies of TNF-receptors and TNF-ligands, and other possible inhibitors of death-inducing effects of physiologic apoptosis, and they considered that, activation or downregulation of apoptosis have a pathogenetic role in the outcome of granulomatous disorders.[7]

Kunitake et al. found increased numbers of cells going into apoptosis in lung tissue of patients with sarcoidosis compared to controls. They used TUNEL method for analysing apoptotic cells.[8] Similar results were obtained by Dai and Domagala.[9,10] They found increased levels of expression (Fas and/or TNF-R1) on T-lymphocytes in bronchoalveoler lavage (BAL) fluid suggesting increased apoptosis. Rutherford et al determined that increased apoptosis by TNF pathway clearly had a pro-survival profile in year 2001.[11]

In contrast, Stridh et al. found no apoptotic morphology in BAL fluid lymphocytes from patients with sarcoidosis. They suggested that BAL

lymphocytes from sarcoidosis patients were resistant to induction of apoptosis and this mechanism might partially explain the accumulation of inflammatory cells and persistence of chronic inflammation.[12] Similarly, Xaus et al. showed that macrophages were unable to undergo apoptosis and found no difference between pro-apoptotic and anti-apoptotic genes.[13]

All of the studies in the literature used different methods for detecting apoptotic cells, because the apoptotic index of a population of cells can be measured by a wide variety of techniques. Detailed and accurate characterization of apoptotic cells based on morphologic hallmarks such as nuclear condensation and fragmentation, membrane blebbing and cell shrinkage can be made using microscopy. The most commonly used technique to quantify apoptosis in fixed tissues is the terminal transferase-mediated dUTP nick end-labeling (TUNEL) method. TUNEL is a common method for detecting DNA fragmentation that results from apoptotic signaling cascades. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase (TdT), an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. It may also label cells that have suffered severe DNA damage.[15]

In our study, we used TUNEL method to evaluate apoptosis. We found that apoptosis was higher in low stage disease than high stage disease. Since few number of patients with high stage disease is included in our study, our findings should be supported with further studies, which should include a higher number of patients with high stage disease.

In conclusion, apoptosis might lead to the death of disease-initiating, auto-reactive T cells in granulomas and play a critical role in granuloma resolution and also the presence of apoptotic events in sarcoidosis might correlate with the resolution of granulomatous inflammation and the elimination of the granulomas; which might indicate that the persistence of the granulomas correlates with a reduction of apoptosis. Apoptosis can be mediated by cell surface receptor/ligand interaction (Fas/Fas-L,TNF) or by soluble factors and both positive and negative regulation of apoptosis influences initiation, progression, or healing of tissue damage and the balance of these two regulations may critically influence the inflammatory process. Hence, it would be also important to understand the role of apoptosis in the pathological process of lung injury and repair in order to provide new therapeutic strategies of sarcoidosis management.

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