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Evaluation of caspase-3 and bcl-2 expressions in spleen tissue of streptozotocin-induced diabetic rats by immunohistochemistry and quantitative RT-PCR

Streptozotosine bağlı diabetik ratların dalak dokusunda caspase-3 ve bcl-2 ekspresyonlarının immünohistokimyasal ve kantitatif RT-PCR ile değerlendirilmesi

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Summary

Aim : Spleen tissue is comprised of two functionally and morphologically distinct components, the red pulp and the white pulp. Aim of this study to evaluate the activated apoptotic pathways in spleen tissue of streptozotocin-induced diabetic rats. Apoptotic effects of diabetes in the generated animal model were analyzed in both components.

Material and Methods: The working groups consisted of healthy controls (Group 1, n = 10) and streptozotocin-induced diabetic rats (Group 2, n = 10). Cell morphology and integrity was evaluated by light microscopy using hematoxylin and eosin staining. Apoptosis was determined by immunohistochemical and real-time quantitative polymerase chain reaction (RT-PCR) analysis of Caspase- 3 and Bcl-2.

Results: All results were evaluated statistically. At the end of our study we determined that in the diabetic group fibrotic area contracted, causing distortion of the splenic contour and that the paranchymal fibrosis was sufficiently extensive to involve the marginal zone and follicular areas of the white pulp. In the diabetic group also more cells activated the process of apoptosis by increasing Caspase-3 and suppressing Bcl-2 expression when analyzed immunohistochemically. In agreement mRNA expression of Caspase-3 was higher in the diabetic group compared to that of the control group, whereas mRNA expression of Bcl-2 was significantly lower determined.

Conclusion: These findings suggest that in spleen diabetes has an important influence on apoptotic pathways through Caspase-3 and Bcl-2.

Key Words: Apoptosis, Bcl-2, Caspase-3, diabetes mellitus, experimental.

Özet

Amaç : *Dalak dokusu iki fonksiyonel ve farklı yapıdan bir araya gelmektedir, kırmızı pulpa ve beyaz pulpa. Bu çalışmanın amacı streptozotocin ile indüklenmiş diyabetik sıçanlarda dalak dokusundaki aktive olan apoptotik yolakları araştırmaktı. Oluşturulan hayvan modelinde diyabetin apoptotik etkileri her iki yapıda araştırıldı.*

Gereç ve Yöntem : *Çalışma grupları sağlıklı kontrollerden (Grup 1, n = 10) ve streptozotocin ile indüklenmiş diyabetik sıçanlardan (Grup 2, n = 10) oluşmaktaydı. Hücre morfolojisi ve bütünlüğü ışık mikroskobu altında hematoksilen ile eozin boyası ile değerlendirildi. Apoptoz, Caspaz-3 ve Bcl-2' nin immunohistokimyasal ve gerçek-zamanlı kantitatif polimeraz zincir reaksiyon (RT-PCR) analizleriyle belirlendi.*

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Bulgular: *Tüm sonuçlar istatistiksel olarak değerlendirildi. Çalışmamızın sonucunda diyabetik grupta fibrotik alanın daralarak, dalak kontüründe bir bozukluk meydana getirdiği ve parankimal dokunun yaygın olarak genişleyerek beyaz pulpanın marjinal zonunu ve foliküler alanını kapsadığı gözlendi. Diyabetik grup immunohistokimyasal olarak analiz edildiğinde ayrıca daha fazla hücrenin Caspaz-3'ü arttırarak ve Bcl-2 ekspresyonunu baskılayarak apoptotik süreci aktive ettiği izlendi. Bununla uyumlu şekilde diyabetik gruptaki Caspaz-3 mRNA ekspresyonu kontrol grubuna göre artmış, Bcl-2 mRNA ekspresyonu ise anlamlı şekilde azalmış oldukları bulundu.*

Sonuç: *Bu bulgular diyabetin dalakta Caspaz-3 ve Bcl-2 üzerinden apoptotik yolaklar üzerinde önemli etkileri bulunduğunu düşündürmektedir.*

Anahtar Kelimeler: *Apoptozis, Bcl-2, caspase-3, diabetes mellitus, deneysel.*

Introduction

Diabetes mellitus is a heterogeneous primary disorder of carbohydrate metabolism with multiple etiologic factors that generally involve absolute or relative insulin deficiency or insulin resistance or both (1). Functions of the spleen are centered on the systemic circulation. As such, it lacks afferent lymphatic vessels. It is comprised of two functionally and morphologically distinct compartments, the red pulp and the white pulp. The red pulp is a blood filter that removes foreign material and damaged erythrocytes. It is also a storage site for iron, erythrocytes, and platelets (2). In rodents, it is a site of hematopoiesis, particularly in fetal and neonatal animals.The spleen is also the largest secondary lymphoid organ containing about one-fourth of the body's lymphocytes and initiates immune responses to blood-borne antigens (3,4). This function is charged to the white pulp which surrounds the central arterioles. The white pulp is composed of three subcompartments: the periarteriolar lymphoid sheath (PALS), the follicles, and the marginal zone (2). Streptozotocin (STZ), which is commonly used to create experimental diabetic animal models, is a component that leads to the inhibition of pancreatic insulin secretion and tyrosine kinase activation by decreasing insulin receptors on target tissues **(**5,6). We used streptozotocin to induce diabetes in rats and to evaluate the apoptotic pathways in spleen activated by this disease. Apoptosis can be triggered by 3 mechanisms: The binding of Fas ligand (Fas-L) to Fas receptor expressing cells (7, 8), the activation of Bcl-2 family members (9,10) and the endoplasmic reticulum pathway (11, 12). Cell destruction occurs through the activation of caspases, a highly conserved family of cysteine proteases that have specificity for aspartic acid residues in their substrates (13, 14, 15). Caspase-3 is an executioner, an 'expressive' caspase that has been implicated as a key protease cleaving multiple cellular substrates, including components related to DNA repair and regulation, to bring the cell to its demise. Activation of Caspase-3 also leads to proteolytic cleavage of other caspases and endogeneous protein substrates. Measurement of Caspase-3 allows the detailed study of apoptotic mechanisms (16). A well studied apoptosis promoting member of the Bcl-2 gene family is *Bax*. Bcl-2 is known to form heterodimers with the Bax protein *in vivo* and the molar ratio of Bcl-2 to Bax determines whether apoptosis is induced or inhibited in target tissues (17). The Bax protein is considered to be one of the primary targets of p53 and controls cell death through its participation in the disruption of mitochondria with the subsequent release of cytochrome c (18). Cytochrome c release in turn activates caspases 9 and 3 (19). Cleaved Caspase-3 (p17 kDa and p19 kDa) is regarded as a proximate mediator of apoptosis. Excessive reactive oxygen species (ROS) activate the process of apoptosis by increasing Caspase-3 activity and suppressing *Bcl-2* expression (20, 21). This study was composed to evaluate the effects of diabetes on the above mentioned apoptotic pathways in the lymphoid tissue structure of spleen by light microscopy, immunohistochemistry and quantitative RT-PCR.

Materials ans Methods

Animals; The study protocol complies with the European Community guidelines for the use of experimental animals and all experiments were approved by the Animal Ethics Committee at Ege University School of Medicine (2009-16).Twenty *Rattus albinus* weighing 200-250 g were selected for this study. All animals were housed at 23 ± 2 ℃ under 12-h light and dark cycle s, and allowed access to food and water ad libitum. Animals were randomly divided into two groups: controls (n = 10) and diabetic rats (n = 10). Diabetes was induced by a single intraperitoneal injection of STZ (55 mg/kg body weight) dissolved in 0.1 mol/l sodyum sitrate buffer (pH 4.7). 24 and 48 hours after administration of STZ, the tail vein blood glucose level was measured in all animals. Blood glucose levels of 250 mg/dl and above were considered diabetic. At the end of two months blood glucose level weas measured again.

Histological and Immunohistochemical Procedures*;* At the end of the observational and experimental time

animals were sacrificed. Their spleen was removed, Following fixation, were washed in 0.1M phosphate buffer solution (PBS) and dehydrated through graded ethanol series, cleared in xylene before embedding in paraffin. Paraffin blocks were cut into 5 *µ*m serial sections (Leica RM 2145), stained with hematoxylin and eosin (H&E) and examined by light microscopy. For immunohistochemical analyses again 5 µ thick cross-sections were used for the activated Caspase-3 and Bcl-2 detection system [Biovision activated Caspase-3 (1:100) and Bcl-2 (1:100)]. In brief, the deparaffinization procedure was accomplished in Xylene for 1 h. Rehydration was done in 100 %, 95 %, 80 % and 70 % alcohol series for 2 min each. After leaving in distilled water for 5 min, the tissues samples were marked on the object slide, washed in phosphate buffer solution (PBS) for 10 min and than left in trypsin for 15 min. Then the primary antibody was applied in an incubator at 57°C and washed with PBS. Afterwards the biotinylated secondary antibody was applied, washed with PBS before incubating with the enzyme conjugate and 3,3-diaminobenzidine tetrahydrochloride (DAB). The whole procedure was finished after staining the sections with Mayer's hematoxylin (Zymed Laboratories, USA).

RNA preparation and real-time quantitative RT-PCR*:* Total RNA was prepared from rat spleen tissues using the TriPure Isolation Reagent (Roche Applied Science). 10 µg of total RNA was then reverse transcribed with Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Primer and probe sets were designed using the Universal Probe Library (Roche Applied Science; Cat. Nr. Bcl-2: 04689038001, Caspase-3: 04689046001, G6PDH: 04688554001). TaqMan RT-PCR analysis of rat tissues was performed on the LightCycler v.2.0 instrument. Quantification of mRNA was achieved by using known serial dilutions of GAPDH standards included in each run. Expression levels were normalized and represented as the logarithmic ratio of Bcl-2 and Caspase-3 mRNA values to GAPDH mRNA.

Results

Histological and Immunohistochemical Findings: In diabetic rats the fibrotic area contracted causing distortion of the splenic contour. Parenchymal fibrosis was sufficiently extensive to involve the marginal zone and follicular areas of the white pulp. Focal areas representing hemorrhage could be detected. Both red and white pulps were replaced by fibroblasts and scar tissue. Also, the contours of the white pulp were lost and appeared morphological irregular in the diabetic group when compared with the control group (Fig. 1 A-B).

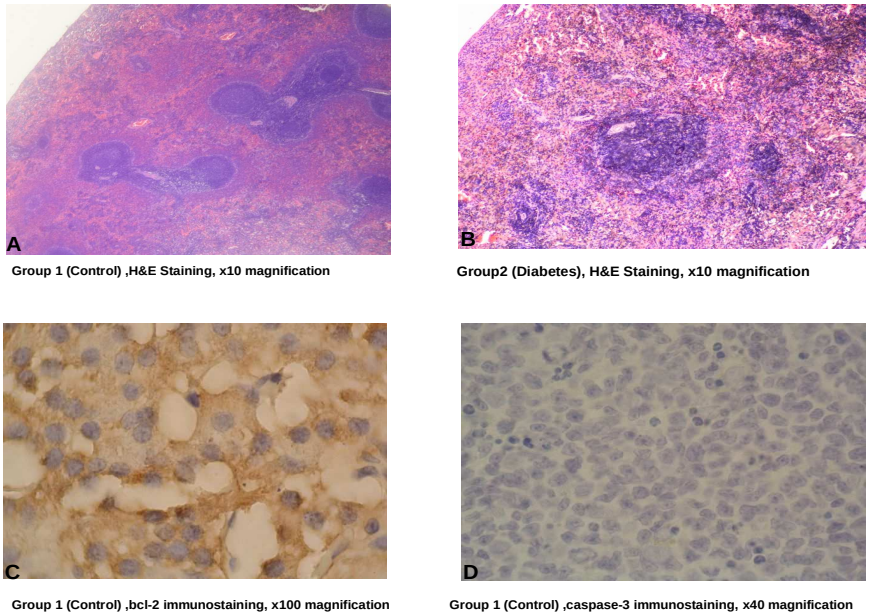


Figure1. The contours of the white pulp were lost and appeared morphological irregular in the diabetic group when compared with the control group (Fig. 1 A-B).The immunoexpression of Bcl-2 was extensive and that of Caspase-3 low in the control group, whereas it was the opposite in diabetic group (Fig. 1 C-D)

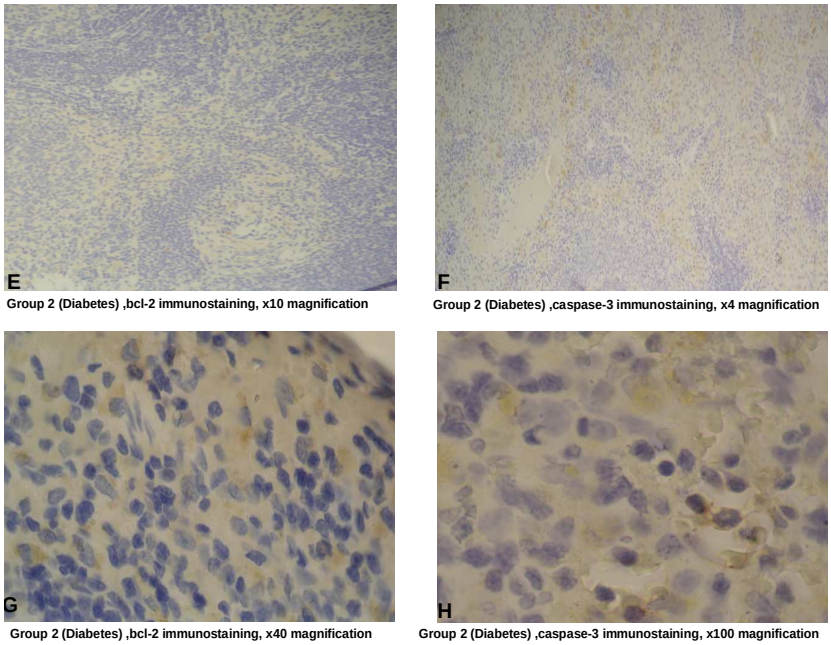
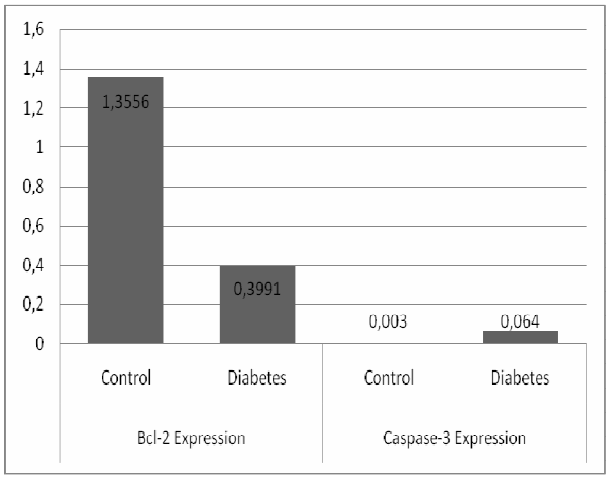


Figure 2: Diabetic group (Fig. 2 E-F-G-H). Apoptosis could also be characterized by chromatin condensation and karyorrhexis of lymphocytes. In apoptotic cells the Caspase-3 immunoexpression was clearly increased (x) by suppressing Bcl-2 expression (Fig. 2 G-H).

Whereas the immunoreactivity of Bcl-2 was extensive and that of Caspase-3 low in the control group (Fig. 1 C-D), it was the opposite in diabetic group (Fig. 2 E-F-G-H). Apoptosis could also be characterized by chromatin condensation and karyorrhexis of lymphocytes. In apoptotic cells the Caspase-3 immunoreactivity was clearly increased by suppressing Bcl-2 expression (Fig. 2 G-H).

RT-PCR Findings: The average of Bcl-2 expression was 1,3556 ± 0.109 versus 0.399 ± 0.183 and Caspase-3 expression was 0.003 ± 0.004 versus 0.064 ± 0.018 in the control and diabetic groups, respectively (Table 1). We found that Bcl-2 mRNA expression was significantly higher and Caspase-3 expression was significantly lower in the control group (Graph 1).



Graph 1: Comparison of Caspase 3 and Bcl-2 expression levels in control and diabetes.

Table 1: Bcl-2 and Caspase–3 expression levels in control and diabetes groups.

	GROUPS	Mean	SD*
Bcl-2 Expression	Control	1,3556	0,109
	Diabetes	0,3991	0,183
Caspase-3 Expression	Control	0,0030	0,004
	Diabetes	0,0640	0,018

*Standart deviation ±

Discussion

International Diabetes Federation reported that at least 177 million people in the world have diabetes and World Health Organization figures estimate that this will rise to 300 million by 2025 (22). Diabetes mellitus is a multifaceted syndrome and attacks many organ systems including the spleen. Recent biomedical and genetic studies have substantially increased ourunderstanding of cell cycle and cell death signal transduction pathways, increasing potential targets for the treatment of proliferative disorders. Despite of these researches, the

mechanism(s) activating apoptotic pathways in the diabetic spleen have not been completely understood. In the present study, we showed the effects of hyperglycemia on the expression of Bcl-2 and Caspase-3 by immunohistochemical staining in the spleen of STZ induced diabetic rats and at the mRNA level by quantitative RT-PCR. We focused on two key proteins in apoptosis, the cellular death mechanism: Caspase-3, a protease cleaving multiple cellular substrates and Bcl- 2, an anti-apoptotic protein. Apoptosis involves the orderly breakdown and packaging of cellular components and their subsequent removal by surrounding structures. After apoptosis has been initiated, a complex series of second messengers and cell-death–specific proteins become activated. Mitochondrial-dependent apoptosis has been shown to require release of cytochrome c from mitochondria and subsequent activation of a specific class of cytoplasmic proteases known as caspases. Cysteinyl aspartate-specific proteases (caspases) have been identified as key players in the cellular process termed programmed cell death or apoptosis. The caspase family of enzymes contains cysteine proteinases that mediate a whole range of intracellular events, and a number of agents act by modulating caspase expression and activity directly, bypassing the receptor pathway (23). There are presently 13 known caspase family members, which form an intracellular proteolytic cascade that modulates many cellular events in the apoptotic pathway, including activation of transcription factors (24). Caspase-3 activation has been observed to induce cell death without DNA fragmentation, but apoptosis without Caspase-3 activation involvement has been also observed. As well as autoimmune diseases, beholding the proliferative retinal disorders showed that cell proliferation and apoptosis appear to be key mechanisms regulating certain cell populations in epiretinal membranes of proliferative vitreoretinopathy, proliferative diabetic retinopathy and macular pucker (16). Inhibition of proliferative regulators such as PCNA and/or activation of apoptotic executors such as Caspase-3 may serve as therapeutic targets to halt progression of proliferative retinal disorders (16). Bcl-2 which could be localized to mitochondria, has been shown to inhibit cytochrome c release and protect against oxidative stress-induced apoptosis. Yang *et al.* showed that cytosolic cytochrome c is necessary for the initiation of the apoptotic program, suggesting a possible connection between Bcl-2 and cytochrome c, which is normally located in the mitochondrial intermembrane space (25). In this study, we were able to reveal that the decreased Bcl-2 level causes widespread apoptosis in the lymphoid tissues in the diabetic group. Also discussed effects of oxidation and inflammation on apoptotic response of vascular cells(26). A similar questioning of major function of spleen tissue is also in question in our work. Immune dysfunction is associated with increased oxidative damage and mitochondrial dysfunction and that the nutrient treatment effectively elevated immune function, decreased oxidative damage, enhanced mitochondrial function, and inhibited the elevation of apoptosis factors (27). Our study out the factors for formed immune dysfunction in diabetes and activation of Caspase-3 in the diabetic group.Immunohistochemical studies revealed that, in mirror sections, the staining of Bax and activated Caspase-3 were observed in the TUNELpositive cell area, but the expression of Bcl-2 in these apoptotic cells was generally too low to be detected (28). These results suggest that a Bax-regulated mitochondrial cytochrome c mediated Caspase-3 activation pathway might be involved in the diabetic tissues. There is not enough data about Bcl-2 and Caspase-3 mRNA expression level in the diabetic spleen. We found significantly lower Bcl-2 mRNA expression and higher Caspase-3 expression in the diabetic spleen tissues than normal and showed that diabetes causes activation of Caspase-3 activity that is associated with Bcl-2-inhibition. Molecular and histological results support each other and provide an increased understanding of the mechanisms by diabetes dependent damages of lymphoid tissues in this study.

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