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Quantitative approach to lectin-based glycoprofiling of thymic tissues in the control- and the dexamethasone-treated mice

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ABSTRACT

Dexamethasone (DEX) is the most commonly used synthetic glucocorticoid in treatment of various inflammatory conditions. Here we focused on evaluating the effect of DEX on apoptosis and glycan profile in the mouse thymic tissues. Histological examinations revealed that the DEX treatment cause severe alterations in thymus, such as disruption of thymic capsule, impaired epithelial cell-thymocyte contacts, cellular loss and increased apoptosis. The identification of thymic glycans in the control- and the DEX-treated mice was carried out by using a panel of five plant lectins, *Maackia amurensis* agglutinin (MAA), peanut agglutinin (PNA), *Sambucus nigra* agglutinin (SNA), Concanavalin A (ConA) and wheat germ agglutinin (WGA). Lectin histochemistry results showed that glycosylation pattern of thymus changes upon DEX treatment. For further detailed quantitative analyses of the binding intensities for each lectin, histochemical data were scored as high positive (HP), mild positive (MP) and low positive (LP) and differences among signaling densities were investigated. The staining patterns of thymic regions observed with lectin histochemistry suggest that DEX can affect the thymic glycan profile as well as thymocyte apoptosis. These results are consistent with the opinion that not only sialic acid, but also other sugar motifs may be responsible for thymocyte development.

1. Introduction

Proper education of thymocytes and subsequently generation of a functional T cell repertoire are vital processes to form effective immune responses (Miller, 1961). In the intrathymic microenvironment, interaction of developing thymocytes with an epithelial network is more essential for the phenotypic changes of thymocytes and proliferation of them to functional T cells having both immunological tolerance to self and being reactive to foreign stimuli. During the T cell development, numerous cellular and stromal molecules are temporally and spatially controlled (Patel and Haynes, 1993; Singer and Haynes, 1987). At each step of intrathymic development, thymocytes present different glycan (the glycoconjugate repertoire of glycoproteins and glycolipids) profiles on their surfaces. For example, immature cortical thymocytes bind PNA lectin, which is probe for non-sialylated core 1 type *O*-glycans [Galactose (Gal)β1,3*N*-acetylgalactosamine (GalNAc)], whereas mature cortical ones have an affinity to specific lectins such as MAA and SNA, which are particularly recognize α2,3- and α2,6-linked sialic acids (SAs), respectively (Baum et al., 1996; De Maio et al., 1986). ConA, the first lectin isolated by affinity chromatography, probably the most widely used lectin due to its notable biological features, for instance it binds only to some specific *N*-glycans in animals (Cummings and Etzler, 2008; Goldstein and Poretz, 1986). WGA, another commonly used lectin, binds to SA with an affinity fourfold than GlcNAc (Bhavanandan and Katlic, 1979). Therefore, WGA lectin seems to be a useful tool for fractionation of T cells as previously reported (Boldt and Lyons, 1979; Hellstrom et al., 1976). Since these lectins are commonly used to identify their specific glycans, a more detailed understanding of their specificities in thymic microenvironment would be valuable.

Reengineering of the cellular and environmental glycan profile can affect the thymocyte-thymic stroma interactions, thereby T cell functions by modulating differentiation, migration and apoptosis of developing thymocytes (Baum, 2002; Baum et al., 1996; Baum et al., 1995; Galvan et al., 2000; Perillo et al., 1995; Yang et al., 1996). It is well established that cell surface carbohydrates have a potential for encoding biological information and mediate fundamental cellular events such as cell–cell and cell-matrix interactions as well as cell proliferation and apoptosis, a key molec-

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ular event in selecting the self-tolerant T cell repertoire during the intrathymic development (Brandley and Schnaar, 1986; Varki and Gagneux, 2012). Although the signaling events can trigger the apoptotic pathways during negative and positive T cell selection, the roles of stromal and cell surface carbohydrates on T cell death were not fully understood. It is commonly accepted that glucocorticoids, the widely used anti-inflammatory drug, have essential immunoregulatory effects on differentiation, trafficking, and apoptosis of thymocytes (Cohen, 1992; Gruber et al., 1994; McConkey et al., 1990; Savino et al., 2016; Wyllie, 1980; Zacharchuk et al., 1990). In thymic microenvironment, immature $CD4^+CD8^+$ double positive cortical thymocytes expressing abnormal or unrelated T cell receptor (TCR) profiles are major targets for glucocorticoid-induced apoptosis (Herold et al., 2006; Lechner et al., 2000; Vacchio et al., 1994; von Boehmer and Kisielow, 1990; Zilberman et al., 1996), unless saved by TCR-dependent stimuli (Ashwell et al., 2000). Immunoregulatory effects of glucocorticoids in thymus have been mediated by ligand-activated glucocorticoid receptors, which are required for induction of "death genes" in the selection process denoted "death by neglect" (Chung et al., 2002; Cifone et al., 1999; Wang et al., 2006). Although extensive researches have been revealed that many genes are altered upon the glucocorticoid stimuli, which are essential for activation, function and apoptosis of thymocytes, including *Bcl-X_L* (Gascoyne et al., 2003), IκBα (Deroo and Archer, 2001), *GILZ* (D'Adamio et al., 1997), *GITR* (Nocentini et al., 1997) *Itk*, *Txk* and *Lck* (Petrillo et al., 2014), the mechanism underlying glucocorticoid-induced thymocyte apoptosis has not been well understood yet.

Despite the existence of some findings which described the glucocorticoids-induced regulation of molecules in the thymic microenvironment (Lannes-Vieira et al., 1991a,b), surfaces of the thymic epithelial cells (De Souza and Savino, 1993) and thymocytes (Van Laethem et al., 2001), there is no information on the effect of glucocorticoids on glycans in the thymic microenvironment. The present study aims at determining whether the glycan profile of the thymus is affected by exogenous glucocorticoid, dexamethasone (DEX) and ascertaining whether changes of cell surface glycan profile correlate with apoptosis in DEX-treated mice.

2. Materials and methods

2.1. Reagents

DEX was purchased from I.E. Ulagay (Istanbul, Turkey). Digoxigenin-labeled lectins, PNA, MAA and SNA, anti-digoxigenin alkaline phosphatase, 3,3′-diaminobenzidine (DAB), 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP), TUNEL assay kit and proteinase K were obtained from Roche Applied Sciences (Mannheim, Germany). Peroxidase-conjugated lectins, ConA and WGA, and other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Ethics statement

The prominence of the thymus in the immune system was first realized through studies on mice. Thus, our knowledge of the intrathymic development arises from the mice. During the first 3th to 4th weeks of life, the thymus continues to develop in male mice. Between 5th to 7th weeks, it completely maturated. However, the ages of 8th to 12th weeks are critical for thymic involution. Finally, a progressive regression in thymus starts at 6th to 8th months (Dominguez-Gerpe and Rey-Mendez, 1998; Sharma et al., 2013). In the present study, 8-week-old mice were chosen as representatives of fully mature thymic microenvironments.

All experiments were carried out with 65 male Balb/c mice, weighing 25–33 g, and have been approved by the Animal Experimentation Ethics Committee of the Medical School, Celal Bayar University, Manisa, Turkey. Mice were allowed ad libitum access to food and water and maintained on a 12-h light-dark cycle at 22 °C. Due effort was done to minimize suffering and limit the number of animals used. Animal care was ensured in compliance with the previously outlined rules.

2.3. Preparation of control and dexamathasone treated groups

A single intraperitoneal injection of DEX at a dose of 7.5 mg/kg was carried out to thirty-five mice. The dose of DEX and duration of treatment, appropriate for induce apoptosis, were chosen in accordance with previous studies (Chen et al., 2004; Nieto et al., 1992). Thirty control mice received the same volume of 0.9% NaCl isotonic solution under the same condition. After 24 h, the animals were sacrificed by cervical dislocation and the thymus glands were quickly harvested. Some of the thymus glands were fixed with neutral formalin for 24 h, dehydrated with graded ethanol series and embedded in paraffin according to standard procedure for light microscopy. Serial sections of 5 μm thickness were mounted on poly-L-lysine coated slides for Haematoxylin-Eosin (H&E) staining, TUNEL assay and lectin histochemistry.

2.4. TUNEL assay

For the detection of apoptotic cells in thymic tissues, *in situ* Cell Death Detection Kit, POD (Roche Applied Science, Cat # 11684817910) was applied on paraffin sections according to the manufacturer's instructions with slight modifications. Briefly, shortly after the deparaffinization and rehydration, the sections were washed in PBS for 5 min, and incubated with proteinase K (20 μg/ml in 10 mM Tris; Roche Applied Science, Cat # 03 115 836 001) for 20 min at room temperature. Following the washing in PBS, in order to quench endogenous peroxidase activity 3% H_2O_2 in PBS was applied for 5 min and the slides were incubated with TUNEL reaction mix (labeling solution and TdT enzyme) for 1 h in humidified chamber at 37 °C. For negative control, only labeling solution was used. After the washing in PBS for 5 min, the sections were incubated with converter-POD in a humidified chamber for 30 min at 37 °C. Visualization of the positive reaction was accomplished using a 50 μl 0.03% DAB solution. Finally, the slides were counterstained with 0.5% methyl green solution. Micrographs at 200 × magnification were captured as JPEG format (resolution of 2207 × 1704 pixels) using Olympus® BX43 microscope (Tokyo, Japan) and Nikon® Coolpix E4500 digital camera system (Tokyo, Japan). The number of TUNEL-positive cells was counted in randomly selected twenty-five cortical and medullar fields (1063 × 827 pixels, 300 dpi) from each control and DEX-treated sections by using "cell counter" plugin of the ImageJ software, version 1.46c (NIH, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/). Results were calculated as the "% apoptotic index (AI)" using the following formula: the number of apoptotic cells/total cells × 100%. The staining intensity was evaluated by a semi-quantitative scoring system and scored as low (+; >5% AI), mild (++; 6–15% AI), positive (+++; 16–50% AI) and severe (++++; 51 and higher% AI).

2.5. Glycoprofiling

To evaluate glycosylation patterns in the thymus of control and DEX-treated mice five digoxigenin-labeled and peroxidase-conjugated plant lectins, MAA, PNA, SNA, ConA and WGA, were chosen, because they show distinct binding specificities in biolog-