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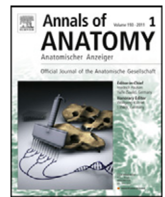

Research article

Immunohistological analysis of cell cycle and apoptosis regulators in thymus

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SUMMARY

The combined expression patterns of cell cycle and apoptosis regulators have not been analyzed in details in human thymus to the best of our knowledge. Our objective was to provide multiparametric and combined immunohistological information regarding the expression levels and the topographical distribution of major cell cycle and apoptosis regulators in postnatal human thymus. Ki67 and cyclins A, B1, D3 and E were frequently expressed by thymocytes with higher expression in cortical than medullary thymocytes. The expression of cyclin D2 was low in thymocytes. Thymic epithelial cells (TEC) exhibited low expression of Ki67 and cyclins. Bid was frequently expressed by thymocytes, Bcl-xL by cortical thymocytes and Bcl-2 by medullary thymocytes. The expression levels of Bim and survivin in thymocytes were low. The expression levels of Bax and Mcl-1 were higher in medullary than cortical thymocytes and TEC. Bak and Bad were mainly expressed in medullary TEC and Hassall Bodies (HB). c-FLIP and Fas were frequently expressed in TEC and FasL was mainly expressed by medullary TEC and HB. Cleaved caspase-3 was expressed by scattered thymocytes at the cortex and the corticomedullary junction and very rarely at the medulla. The different expression profiles and immunotopographical distribution of cell cycle and apoptosis regulators in thymocytes and TEC indicate that their expression is tightly regulated during thymic cell differentiation and that they are differentially involved in the cell survival/death regulation of thymocytes and TEC. Furthermore, this study indicates decrease of the proliferation and caspase-dependent apoptosis of thymocytes from the cortex to the medulla.

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1. Introduction

Cell cycle and apoptosis regulators play crucial roles in normal tissue physiology as well as in pathological processes such as oncogenesis and inflammation (Malumbres and Barbacid, 2001; Igney and Krammer, 2002; Okada and Mak, 2004). Cell cycle regulation relies on a complex molecular network involving cyclins such as cyclins D1, D2, D3, E, A and B1-2, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs) such as p16, p21 and p27 (Malumbres and Barbacid, 2001; Sherr and Adams, 2004). Apoptosis can be initiated by two alternative convergent pathways, the extrinsic pathway, which is mediated by cell surface death receptors and the intrinsic pathway, which is mediated by mitochondria (Igney and Krammer, 2002; Okada and Mak, 2004). In both pathways, cysteine aspartyl-specific proteases (caspases) that cleave cellular substrates are activated resulting in the characteristic morphological and biochemical alterations of apoptosis. However, caspase-independent apoptosis has also been identified (Okada and Mak, 2004). Important roles in the regulation of apoptosis play the bcl2 family proteins, the c-FLIP proteins and the Inhibitors of Apoptosis (IAP) family proteins (Igney and Krammer, 2002; Youle and Strasser, 2008). The bcl2 family comprises (a) anti-apoptotic members (e.g., Bcl-2, Bcl-xL, Mcl-1), (b) pro-apoptotic multidomain members (e.g., Bax, Bak) and (c) BH3-only members (e.g., Bad, Bik, Bim, Bid) which bind to the anti-apoptotic members to promote apoptosis. The c-FLIP proteins interfere with the death receptor pathway by inhibiting the activation of procaspase 8. The IAP family proteins (e.g., XIAP, c-IAP1, c-IAP2, survivin) may suppress apoptosis by binding to and inhibiting caspases or may act as E3-ubiquitin ligases, promoting the degradation of the caspases that they bind.

The human thymus supports the production of self-tolerant T-cells with competent and regulatory functions and their migration to the peripheral blood circulation (Nitta et al., 2008; Hernandez et al., 2010). Cellular components of the thymic microenvironment such as thymic epithelial cells (TEC), dendritic

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cells and macrophages play essential roles in thymic T-cell differentiation through cell to cell contacts and production of soluble factors (e.g. cytokines, chemokines etc.) (Muller-Hermelink et al., 1997; Nitta et al., 2008; Alves et al., 2009; Derbinski and Kyewski, 2010; Klein et al., 2011). The multiple cellular events occurring during thymic T-cell differentiation involve proteins regulating cell-cycle and apoptosis (Hernandez et al., 2010; Xue et al., 2010). In this respect, immunohistological studies reporting the expression of cell-cycle and apoptosis regulators in normal human thymus provided information for the understanding of thymic histophysiology (Krajewski et al., 1994a, 1995; Chen et al., 1996; Hirabayashi et al., 1997; Mateo et al., 1997; Stefanaki et al., 1997; Doglioni et al., 1998; Engel et al., 1998; Kitada et al., 1998; Penault-Llorca et al., 1998; Moulian et al., 1999; Kanavaros et al., 2001a; Park et al., 2002; Zhou et al., 2002; Pan et al., 2003; Spaulding et al., 2006; Salakou et al., 2007). However, most of the aforementioned studies analyzed a few cell-cycle and apoptosis regulators either in various normal tissues or in control normal thymuses in histopathological studies. Moreover, to the best of our knowledge, the expression patterns of cyclin D2, cyclin D3, cyclin E, Bim, Bid, c-FLIP, cleaved caspase-3 and survivin have not been extensively analyzed so far in normal thymus. On the other hand, the expression patterns of cell-cycle and apoptosis regulators may also provide information about Myasthenia Gravis (MG) and thymic epithelial tumors (TET). Indeed, Bcl-2 is expressed in normal medullary TEC and thymomas while p53 is expressed in normal cortical TEC and thymomas (Stefanaki et al., 1997), thereby supporting the histogenetic classification of TET (Marx and Muller-Hermelink, 2000). Moreover, thymic carcinomas have stronger immunoreactivity for Mcl-1 and Bcl-2 and higher level of p53 expression as compared to other histological subtypes of TET (Chen et al., 1996; Pan et al., 2003). Furthermore, the number of Bcl-2 positive lymphocytes in the medulla was significantly higher in the MG thymic tissue than in the control thymic tissue (Onodera et al., 1996).

Despite numerous studies, a detailed analysis of the combined expression patterns of major cell cycle and apoptosis regulators has never been reported on postnatal human thymus to the best of our knowledge. Therefore, the objective of the present study was to provide multiparametric and combined immunohistological information regarding the expression levels and the topographical distribution of major cell cycle and apoptosis regulators in postnatal human thymus. For the analysis of the cell cycle we selected: (a) the Ki67 because it stains all the cycling cells, (b) the cyclins D1-3 and E because they are important for the passage of cells through the G1 phase and their entry into the S-phase and (c) the cyclins A and B1 because they are important for mitosis (Malumbres and Barbacid, 2001; Sherr and Adams, 2004). For the analysis of apoptosis we selected the Bcl-2, Bcl-xL, Mcl-1 and c-FLIP which are major anti-apoptotic factors and the Bax, Bak, Bad, Bim and Bid which are major pro-apoptotic factors (Igney and Krammer, 2002; Okada and Mak, 2004; Youle and Strasser, 2008). The above factors were selected because the assessment of their differential expression levels permits insight into the balance between anti-apoptotic and pro-apoptotic cell potential. We also analyzed the cleaved-caspase-3 because its detection permits the immunohistological identification of cells undergoing caspase-dependent apoptosis (Dukers et al., 2002; Resendez et al., 2004).

2. Materials and methods

Paraffin sections from 12 histologically normal postnatal human thymuses (3 newborns, 7 children and 2 adolescents) which have been previously analyzed for the immunohistological expression of cytokeratins, neural/neuroendocrine markers, beta-tubulin isotypes and dendritic cell markers (Bai et al., 2008; Papoudou-Bai et al., in press) have been included in the present study.

2.1. Immunohistochemistry

The immunostainings were performed using the Ventana autoimmunostainer and according to manufacturer's protocols and instructions. The immunostained sections were analyzed by a Nikon eclipse 50i microscope. The antibodies used are presented in Table 1. Positive control slides for antibodies used were included in all experiments. They consisted of various types of lymphomas (Hodgkin and B and T-cell non-Hodgkin lymphomas) from our previous studies known to display immunopositive cells for the antibodies used in the present study (Kanavaros et al., 2001b; Bai et al., 2004a,b, 2007). Negative controls with omission of the primary antibody were also included. For evaluation of immunostaining, a continuous score system was adopted by using the 40× objective lens and by counting the immunopositive cells with strong staining in ten fields per section (five in the cortex and five in the medulla). The number of immunopositive cells was divided by the total number of the counted cells and the expression was defined as the percentage of positive cells in the total number of the cells counted. Then, the groups of positivity were determined using cut-off levels as follows: less than 1% (−/+), 1–10% (+/−); 10–25% (+); 25% −50% (++); and more than 50% (+++). To achieve a better identification of cells expressing cell cycle and apoptosis proteins we used (a) morphological criteria (cell and nuclear size and shape), (b) immunostaining on serial sections with pan-cytokeratin for epithelial cells, CD3 for T-cells, CD20 for B-cells, S100, CD11c, CD123 and CD207 for dendritic cells, CD68 and CD163 for macrophages and desmin for myoid cells and (c) double staining of Ki67 with pan-cytokeratin or S100 or CD3 taking into consideration previous studies (Parrens et al., 1998; Kanavaros et al., 2001a; Savchenko et al., 2006; Bai et al., 2008; Varga et al., 2010; Papoudou-Bai et al., in press). The Hassall Bodies (HB) in the present study were classified as juvenile, immature, mature, senescent and lymphocyte-rich according to previous studies (Raica et al., 2005, 2006). The counting of immunopositive cells was performed in all HB subtypes which contained epithelial cells except senescent HB which were of large size without any epithelial cells but with calcified, necrotic material, cellular debris or cystic dilatation.

3. Results

3.1. Immunohistological findings in thymocytes (Table 2)

Ki67 and cyclins A, B1, D3 and E were frequently expressed by thymocytes, with higher expression levels in cortical than medullary thymocytes. The expression levels of cyclin D2 were low in both cortical and medullary thymocytes. The expression levels of Bcl-2, Mcl-1 and Bax were higher in medullary than cortical thymocytes (Fig. 1) whereas Bcl-xL exhibited the opposite expression pattern being frequently expressed by cortical thymocytes and showing lower expression in medullary thymocytes. Bid was frequently expressed by cortical and medullary thymocytes. Bim expression levels were low with higher expression in medullary than cortical thymocytes (Fig. 1). Survivin expression levels were very low with higher expression in cortical than medullary thymocytes. Bak positive cortical and medullary thymocytes were very rare or undetectable. Bad, c-FLIP, Fas and FasL positive thymocytes were not detected. Caspase-3 was frequently expressed whereas cleaved caspase-3 was detected in a few thymocytes. The cleaved-caspase-3 positive cells were scattered and mainly localized at the cortex and the corticomedullary junction whereas only very rare cleaved-caspase-3 positive cells were detected at the medulla.

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