Immunohistochemical expression of p53, p21/waf1, Rb, p16, cyclin D1, p27, Ki67, cyclin A, cyclin B1, bcl2, bax and bak proteins and apoptotic index in normal thymus

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Summary. The immunohistochemical expression of p53, p21, Rb, p16, cyclin D1, Ki67, cyclin A, cyclin B1, p27, bcl2, bax, and bak proteins and the apoptotic index (AI) were investigated in 20 normal thymuses (8 adults, 3 adolescents, 5 infants and 4 newborns). The expressions of Rb, Ki67, cyclin A and cyclin B1 were overlapping, being high in the cortex with a tendency for decreased expression toward the medulla. Apoptotic cells were mainly detected in the cortex and the corticomedullary junction, rarely being present in Hassall's corpuscles. The mean values of Ki67, cyclin A, and cyclin B1 expression in thymuses were 77.2%, 32.2% and 21.4% (newborns), 62.4%, 33.7% and 18.5% (infants), 56.9%, 23.4% and 18.9% (adolescents) and 38.7%, 21.7% and 14.6% (adults), respectively. The mean values of AI in thymuses from newborns, infants, adolescents and adults were 1.4%, 2.9%, 2.7% and 3.8%, respectively. This decrease in proliferation and increase in apoptosis may account for the process of thymic involution. P16 expression was widespread with most of Hassall's corpuscles being p16-positive. P16-positive cells and Hassall's corpuscles increased with the increase in age, in keeping with the suggested role of p16 in cellular senescence. P27 expression was undetectable in subcapsular thymocytes with a tendency for increased expression toward the medulla. The expressions of Ki67, cyclin A and cyclin B1 were inversly related with that of p27, consistent with previous evidence that p27 concentration is reduced when the cell-cycle progresses. P21 and much less frequently p53 proteins were mainly detected in a part of the subcapsular cortical epithelial cells. These findings suggest that a) in thymocytes, the apoptotic pathway is mostly p53-independent and the function of p21 as a negative regulator of the cell cycle must be redundant to other negative regulators, such as p16 and p27 which were abundantly detected in thymocytes and b) in some thymic epithelial cells, the p21 expression may be induced by p53, but in most of them seems to be p53-independent. Most of Hassall's corpuscles were p21-positive, consistent with previous evidence that these structures represent end stages of maturation of thymic medullary epithelium and that p21 protein is involved in the process of terminal differentiation. Cyclin D1 positivity was found in some macrophages. Bcl2 expression was mainly seen in medullary thymocytes, reflecting the surviving thymocytes in this region. The expressions of Bax and bak were more widespread in both the medulla and cortex, suggesting that these proteins play a broader role than bcl2 in the regulation of thymic apoptosis.

Key words: Cell cycle, Immunohistochemistry, Apoptosis, Thymus

Introduction

The regulation of cell cycle and proliferation in normal tissues relies on a complex molecular network involving cyclins, cyclin-dependent kinases (CDKs), cyclin-dependent kinase inhibitors (CDKIs) (Gillet and Barnes, 1998) and two major growth-regulatory pathways: the p53 pathway and the Rb1 pathway (Liggett and Sidransky, 1998; Lopez-Saez et al., 1998; Mulligan and Jacks, 1998; Prives and Hall, 1999).

Cyclins and CDKs form complexes acting at different phases of the cell cycle. Cyclins are divided into two main families (Gillet and Barnes, 1998). The G1 family includes cyclins C, D1-3 and E, which are important for the passage of cells through the G1 phase and their entry into the S-phase. The other family includes the mitotic cyclins A and B1-2. The activity of the cyclin-CDK complexes is negatively regulated by the CDKIs. There are two known families of CDKIs.

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The INK4 family proteins (p16, p15, p18, and p19) inhibit cyclin D-CDK4/6 complexes, while the p21 family proteins (p21, p27 and P57) inhibits all CDKs by binding to cyclin-CDK complexes.

p53 protein may induce cell cycle arrest and/or apoptosis in response to different cellular stress (Prives and Hall, 1999). p53-dependent GI arrest is mediated, at least in part, through p53-mediated induction of the CDKI p21. The activity and the stability of p53 protein is regulated via interactions with proteins such as mdm2 which allows targeting of p53 to the ubiquitin-mediated proteolytic network. Phosphorylation of the Rb protein (pRb) results in release of the E2F transcription factor which is involved in the regulation of S-phase entry (Liggett and Sidransky, 1998; Mulligan and Jacks, 1998). pRb phosphorylation is stimulated by cyclin-D/CDK4 complexes and inhibited by the the CDKI p16. Moreover, there is recent evidence for a complex network linking the function of Rb and p53 genes via mdm2 directly (Hsieh et al., 1999).

There is substantial evidence that the cell cycle and apoptosis are interconnected since cell cycle regulatory proteins may impinge on the apoptosis and vice versa (Brady et al., 1998; Gil-Gomez et al., 1998; Lundberg and Weinberg, 1999). Indeed, besides cell-cycle regulation, p53 is also involved in apoptosis through transcriptional regulation of redox-related genes, the formation of reactive oxygen species and the oxidative degradation of mitochondrial components (Polyak et al., 1997). p53 may downregulate the expression of the antiapoptotic bcl2 gene and upregulate the expression of the proapoptotic bax gene (Cox, 1997). In general, bcl2 and bcl-xl inhibit apoptosis, whereas bcl-xs, bax and bak promote apoptosis. Pro- and anti-apoptotic bcl2 protein family members can heterodimerise and seemingly titrate one another's function, suggesting that their relative concentration may act as a rheostat for the cell suicide program (Adams and Cory, 1998; Evan and Littlewood, 1998).

The human thymus is a lymphoepithelial organ in which T-cells develop during fetal life. After maturation and selection in the fetal microenvironment, T-cells emigrate to peripheral lymphoid tissues (lymph nodes, spleen and gut) and establish the peripheral T-cell repertoire (Haynes et al., 1998; Bodey et al., 1999). Although the thymus exhibits a profound involution during normal postnatal life, thymopoiesis with active T-cell receptor gene rearrangements continues normally within perivascular spaces into late life (Flores et al., 1999). The multiple lymphoepithelial cellular events leading to thymic selection involve proteins regulating the apoptosis and the cell-cycle (Schreiber at al., 1996; Muller-Hermelink et al., 1997; Samms et al., 1999; Rosenheimer-Goudsmid et al., 2000) and previous studies reported the immunohistochemical expression of some of these proteins in normal thymuses which were mostly used as control tissues in pathological studies (Ohta et al., 1995; Onodera et al., 1996; Hirabayashi et al., 1997; Mateo et al., 1997; Sanchez-Beato et al., 1997;

Engel et al., 1998; Penault-Llorca et al., 1998; Villuendas et al., 1998; Nielsen et al., 1999). The immunotopographical distribution of protein networks involved in cell-cycle and/or apoptosis regulation may be useful not only for the understanding of thymic histophysiology but also for the analysis of the histogenetic classification of thymic epithelial tumours (Marx and Muller-Hermelink, 1999, 2000). In this respect, we have previously observed that bcl2 is expressed in normal medullary epithelium and medullary thymomas while p53 is expressed in normal cortical epithelium and cortical and predominantly cortical thymomas (Stefanaki et al., 1997).

Prompted by the above data and since there is paucity of multiparametric, combined immuno-histological information regarding cell cycle and apoptosis in normal thymus, we have investigated the immunotopography of p53, p21, Rb, p16, cyclin D1, p27, Ki67, cyclin A, cyclin B1, bcl2, bax and bak proteins as well as the distribution of apoptotic cells using the TdT (terminal deoxynucleotidyl-transferase)-mediated in-situ labelling (TUNEL) method and conventional histology in 20 morphologically normal thymuses from adults, adolescents, infants and newborns. The aim was to determine the immunotopography of proteins involved in a) the p53 and Rb1 growth control pathways, b) the proliferation, and c) the apoptosis in relation with age.

Materials and methods

Material

Twenty thymuses from 8 adults, 3 adolescents, 5 infants and 4 newborns removed during surgery for reasons other than thymic pathology were retrieved from the files of the Departments of Pathology of Agia Sophia Childrens' Hospital of Athens and Evangelismos Hospital of Athens. None of the individuals had autoimmune disease.

Immunohistochemistry

Immunostainings were performed on formalin-fixed, paraffin-embedded tissue sections by the alkaline-phosphatase/anti-alkaline phospatase (APAAP) or the Streptavidin-Biotin Peroxidase labeled (LSAB) procedures. A pretreatment with microwave oven heating was performed as has been previously described (Kanavaros et al., 2000). Monoclonal antibodies directed against p53 protein (DO-7; Dako SA, Glostrup, Denmark, dilution 1:50), p21/waf1 protein (EA-10; Calbiochem, dilution 1:50), Rb protein (Rb1; Dako, dilution 1:20), proliferation-associated nuclear antigen Ki67 (MIB-1; Immunotech, Marseille, France, dilution 1:20), p16 (F-12; Santa Cruz Biotechnology, California USA, dilution 1:100), p27 (F-8; Santa Cruz, dilution 1:50), cyclin D1 (DSC-6; Novocastra, dilution 1:20) cyclin A (6E6; Novocastra, dilution 1:10), cyclin B1

(7A9; Novocastra, dilution 1:10) and bcl2 (Dako 124, dilution 1:50) were applied. In addition, the polyclonal antibodies bax (Dako, code A3533, dilution 1:500) and bak (Dako, code A3538, dilution 1:250) were also applied. Double staining using cytokeratin or CD45/LCA with p53, p21 and Rb, respectively was performed in some cases as has been described previously (Petit et al., 2001). Positive control slides were included in all cases. They consisted of normal thymuses, thymomas and lymphomas from our previous studies known to be positive for p53, p21, or cyclin D1 (Stefanaki et al., 1997; Kanavaros et al., 2000; Kanavaros et al., 2001). For Rb, p16, bcl2, bax, bak, p27, cyclin A, cyclin B1 and Ki67 internal positive control was required. The immunostaining for cyclin A, cyclin B1 and Ki67 was interpreted by four independent observers (P.K., K.S., D.P., D.A.). For evaluation of immunostaining, a continuous score system was adopted by using the x40 objective lense and by counting the immunopositive cells in 10 randomly selected fields. 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 the total number of the cells counted. Then, the groups of positivity were determined using cut-off levels as follows: less than 5% (+/-); 5 to 25% (+); 25% to 50% (++); and more than 50% (+++).

Tunel

The TdT (terminal deoxynucleotidyl-transferase)-mediated in-situ labelling technique (TUNEL) (Apotag kit, Oncor, Calbiochem) was carried out as previously described (Czader et al., 1996). Internal positive control was required and negative controls (sections without TdT) were included in every staining. The results were interpreted as previously described (Kanavaros et al., 2001) by three observers (P.K., M.B., D.R). Morphologically intact TUNEL-positive cells and apoptotic cells in heamotoxylin-eosin stained slides (defined as cells with condensed, hyperchromatic, ring-like, crescentic or beaded chromatin and often surrounded by a clear halo) were considered as positive

and are referred to as apoptotic cells. Necrotic areas were excluded. The number of apoptotic cells was recorded by using the x40 objective and by counting the apoptotic cells in 10 randomly selected fields, corresponding to a total of 2000 to 3000 cells. The apoptotic index (AI) was determined as the number of apoptotic cells expressed as a percentage of the total number of counted cells.

Statistical analysis

Chi-square test was performed for categorical variables. Pearson's and Spearman's correlation coefficients were used for the assessment of correlation between continuous variables. The results were considered as statistically significant with $p<0.05$. The statistical analysis was performed using the program SPSS 8 for Windows.

Results

Analysis of immunotopography

The immunohistochemical results are summarised in Tables 1 and 2. There was a rather overlapping immunotopographical distribution of the Rb, Ki67, cyclin A and cyclin B1 proteins. The expressions of Rb and Ki67, cyclin A and cyclin B1 proteins were high in the cortex, mainly in thymocytes but also in epithelial cells, with a tendency for decreased expression toward the medulla (Figs. 1-3). Statistically significant positive correlation was found between Rb/Ki67, cyclin A/Ki67 and cyclin B1/Ki67 expression in each age group separately and in all groups analysed together (in all $p<0.01$). p16 expression was widespread in both cortex and medulla with most of Hassall's corpuscles being p16-positive. p16-positive cells and Hassall's corpuscles increased with the increase in age. p27 expression was undetectable in subcapsular thymocytes of the outer cortex but an increase in the frequency of positive cells was observed parallel to the progression toward the medulla. The medullary thymocytes showed generalized

Table 1. Expression of p53, p21, Rb, p16, Ki67, p27, bcl2, bax, bak, cyclin A and cyclin B1 proteins in thymocytes.

|	CORTICAL	MEDULLARY
p53	-	-
p21	-	-
Rb	++/+++	+
p16	++/+++	++
Ki67	++/+++	+
p27	+/++	++/+++
Bcl2	+/-	++/+++
Bax	+	++
Bak	+/++	+/++
Cyclin A	+/++	+
Cyclin B1	+/++	+

Table 2. Expression of p53, p21, Rb, p16, Ki67, p27, bcl2, bax, bak, cyclin A and cyclin B1 proteins in thymic epithelial cells.

	SUBCAPSULAR	CORTICAL	MEDULLARY	HASSAL CORPUSCLES
p53	+	+/-	-	-
p21	++	+	+/-	+++
Rb	++	++	+	-
p16	++	++	++	+++
Ki67	+	+	+	-
p27	++	++	++	-
Bcl2	-	-	++	-
Bax	+	+	++	-
Bak	++	++	++	++
Cyclin A	+	+	+	-
Cyclin B1	+	+	+	-

p27 expression. Cortical and medullary epithelial cells as well as Hassall's corpuscles were p27 positive. There was a rather mutually exclusive immunotopographical distribution of the p27 with the Ki67, cyclin A and cyclin B1 proteins. p21 and much less frequently, p53-positive epithelial cells (Fig. 4) were detected mainly in the cortex, particularly in the subcapsular region, whereas these proteins were undetectable in thymocytes.
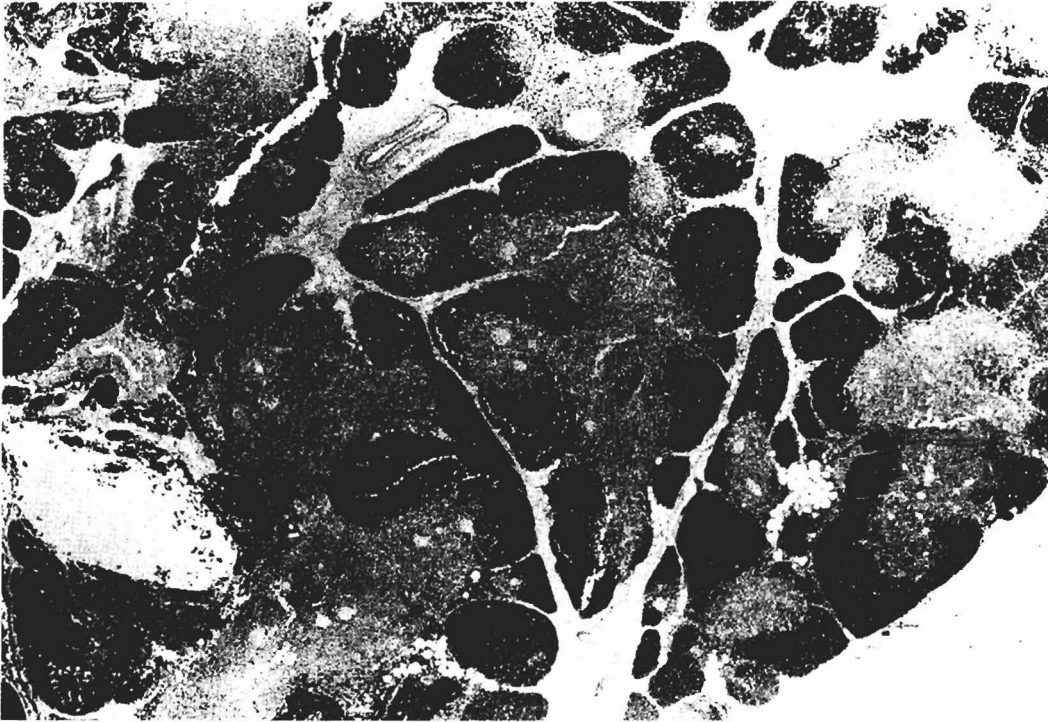

**Fig. 1.** Ki67 immunoexpression in the normal thymus. x 50
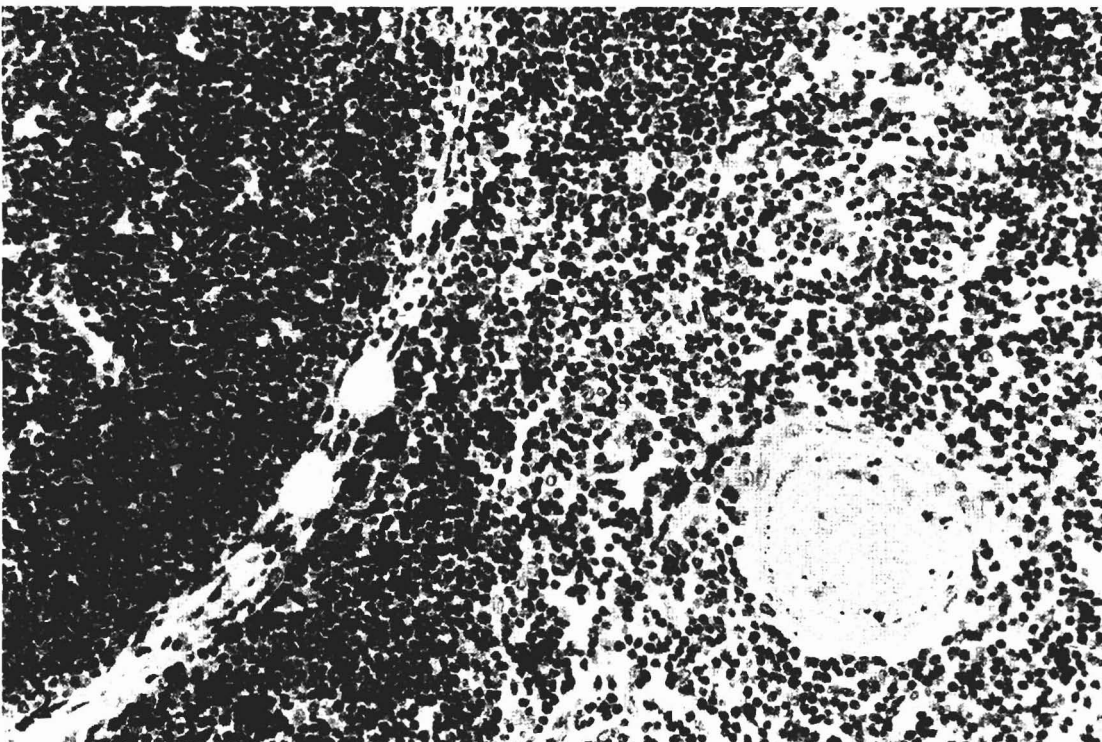
Fig. 2. Cyclin A immunoexpression in the normal thymus. x 250

Although most medullary epithelial cells were p21-negative, Hassall's corpuscles were p21-positive. Cyclin D1 positivity was observed in some macrophages. Bcl2 expression was mainly seen in the medulla and mostly in thymocytes. The expressions of Bax and bak were more widespread, in both the medulla and cortex. Bak showed
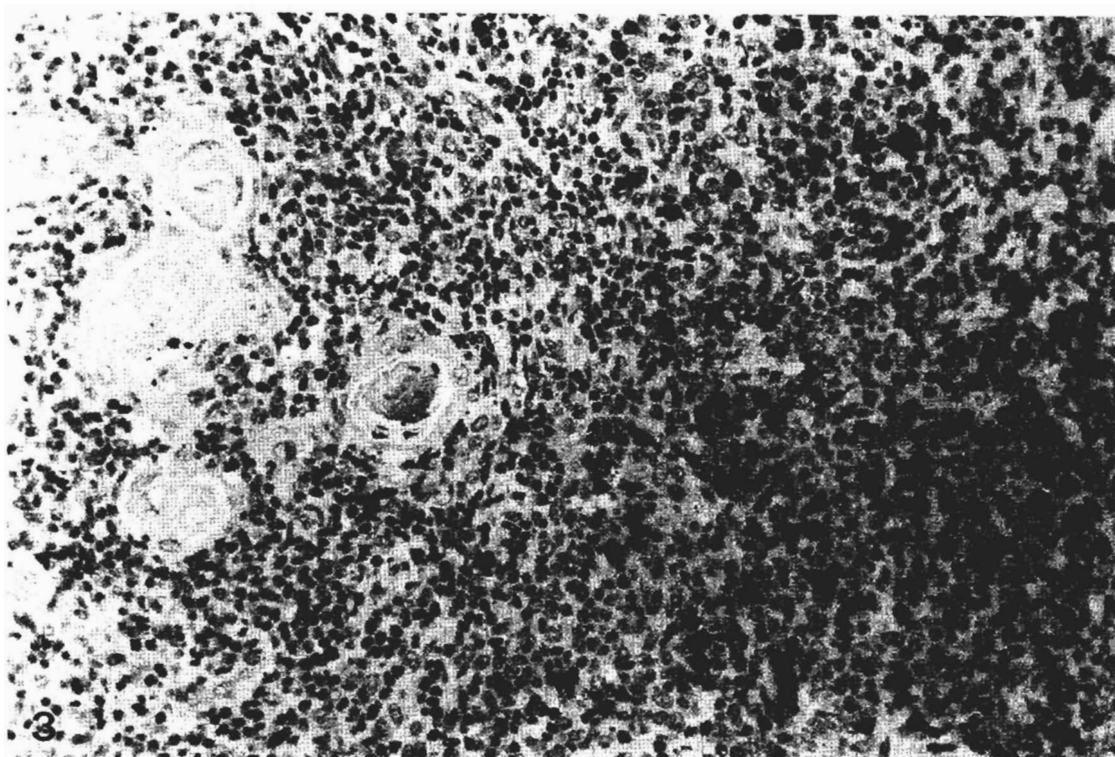
Fig. 3. Cyclin B1 immunoexpression i the normal thymus. x 250
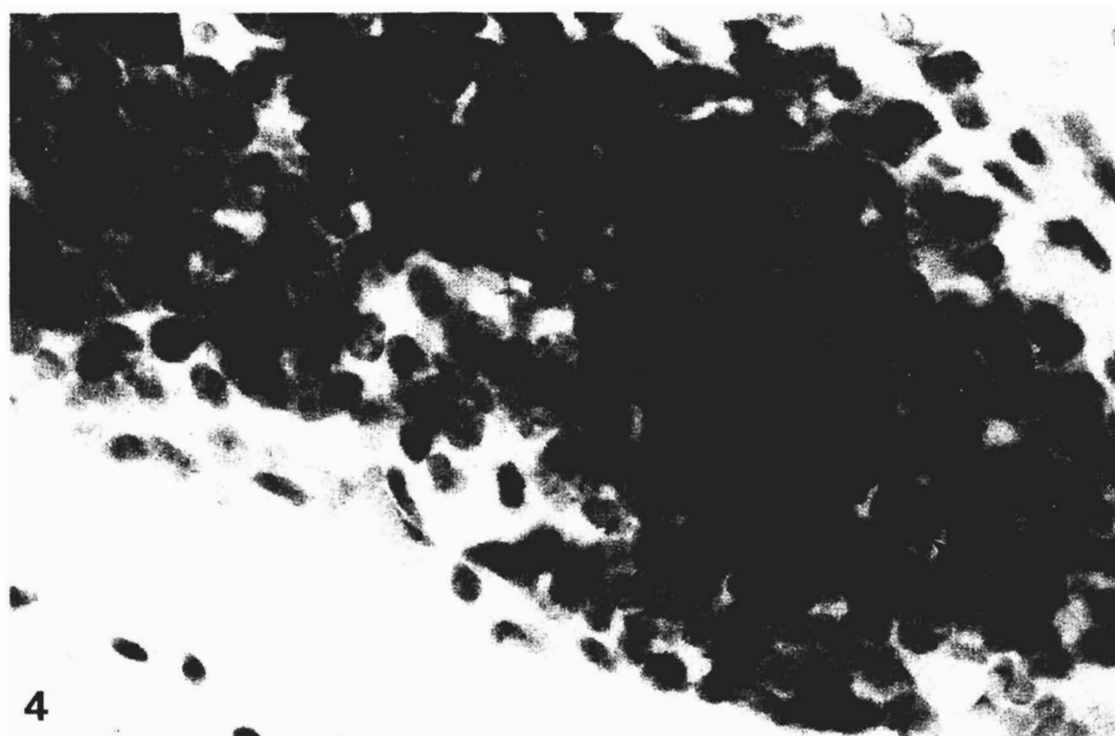
Fig. 4. Double p53 (red staining: arrows) and CD45 (brown staining) immunoexpression in the cortex of normal thymus. x 400

a tendency to be expressed more frequently in epithelial cells than in thymocytes. Hassall's corpuscles were positive for bak but negative for bcl2 and bax. In two thymuses from adults lymphoid follicles were observed. Germinal centers showed high Rb, p16, Ki67, bax and bak expression whereas that of bcl2, p27, p21 and p53 was very low to absent.

Apoptotic index and relation with the proliferation profile

The TUNEL method and the morphological evaluation revealed that apoptotic cells were mainly scattered throughout the cortex and in the corticomedullary junction. Apoptotic cells were rarely detected in Hassall's corpuscles. The mean values of Ki67, cyclin A, and cyclin B1 expression in thymuses were 77.2%, 32.2% and 21.4% (newborns), 62.4%, 33.7% and 18.5% (infants), 56.9%, 23.4% and 18.9% (adolescents) and 38.7%, 21.7% and 14.6% (adults), respectively. The mean values of AI in thymuses from newborns, infants, adolescents and adults were 1.4%, 2.9%, 2.7% and 3.8%, respectively. Statistically significant positive correlation ($p < 0.01$) was found between AI and Ki67 expression in each age group.

Discussion

In the present study, the analysis of the Rb/p16 immunotopography in combination with the proliferation profile showed overlapping immuno-topographical distribution of the Rb and Ki67, cyclin A and cyclin B1 proteins, mainly localised in the cortex. In this region, p16 expression was also high. This tendency for parallel Rb/Ki67 and p16/Ki67 expression has previously been described in normal lymph nodes (Martinez et al., 1993; Villuendas et al., 1997) and indicates that higher levels of Rb and p16 are present in those compartments where cell proliferation is increased, reflecting the roles of Rb and p16 in controlling cell-cycle (Gillet and Barnes, 1998). In addition, high Rb expression may also represent an attempt to inhibit the high rate of apoptosis which occurs in the cortical thymocytes, in view of the evidence that Rb protein may function not only as a negative regulator of proliferation but also of apoptosis (Haas-Kogan, 1995). On the other hand, the expression of p16 was also high in the medulla wherein Ki67, cyclin A and cyclin B1 expression was decreased. This suggests, in keeping with a previous study reporting only on expression of p16 in thymus from newborns (Nielsen et al., 1999), that the expression of p16 does not always correlate with cell proliferation. p16 expression was widespread in both cortex and medulla with most of Hassall's corpuscles being p16-positive. p16-positive cells and Hassall's corpuscles increased with the increase in age. This is in keeping with previous immunohistological results on newborn thymic tissues (Nielsen et al., 1999), showing restriction of p16 staining to Hassall's corpuscles and scattered epithelial and lymphoid cells of the thymus, the only organ comitted to early cellular senescence (Bodey et al.,

1997) and in vitro studies showing that p16 accumulation is associated with cellular senescence in human transformed uroepithelial cells and in malignant glioma cells (Reznikoff et al., 1996; Uhrbom et al., 1997). Taken together, the above data are consistent with the hypothesis that p16 serves a checkpoint function during cellular senescence.

In the present study, the analysis of the p53/p21 immunotopography showed that p21 and p53 proteins were undetectable in thymocytes but p21 and, much less frequently, p53 expression was mainly detected in a part of the subcapsular cortical epithelial cells. These findings suggest that a) in thymocytes, the apoptotic pathway is mostly p53-independent in keeping with previous in vitro data regarding the thymic-epithelial cell-induced apoptosis of CD4+/CD8+ thymocytes (Rosenheimer-Goudsmid et al., 2000) and the function of p21 as negative regulator of the cell cycle must be redundant to other negative regulators, such as p16 and p27 which were detected in a large number of thymocytes, and b) in some thymic epithelial cells, p21 expression may be induced by p53 after cellular stress or DNA damage (Prives and Hall, 1999) and represents an inhibitory response to the cell cycle progression. However, in most of these cells, p21 expression seems to be p53-independent (Cox, 1997). In this respect, it is also noteworthy that a proportion of cortical epithelial cells express Rb protein which was reported to play an essential role in cell cycle arrest after DNA damage (Harrington et al., 1998). On the other hand, most of Hassall's corpuscles were found to express p21 protein. This is consistent with previous evidence that Hassall's corpuscles represent end stages of maturation of thymic medullary epithelium (Bodey et al., 1997) and that p21 protein is involved in the process of terminal differentiation (Cox, 1997).

In the present study, the analysis of the proliferation profile showed that the mean values of Ki67, cyclin A, and cyclin B1 expression in thymuses were 77.2%, 32.2% and 21.4% (newborns), 62.4%, 33.7% and 18.5% (infants), 56.9%, 23.4% and 18.9% (adolescents) and 38.7%, 21.7% and 14.6% (adults), respectively. The mean values of AI in thymuses from newborns, infants, adolescents and adults were 1.4%, 2.9%, 2.7% and 3.8%, respectively. This decrease in proliferation and increase in apoptosis may account for the process of thymic involution.

In the present study, the analysis of apoptosis-related proteins of the Bcl2 family showed Bcl2 expression mainly in the medulla and mostly in thymocytes, reflecting the surviving thymocytes in this region. The expressions of Bax and bak were more widespread, in both the medulla and cortex suggesting that these proteins play a broader role than bcl2 in the regulation of thymic apoptosis. Interestingly, Hassall's corpuscles showed positivity for bak but not for bcl2 and bax. Since bak promotes apoptosis, our findings are in line with previous evidence that Hassall's corpuscles are considered to be the only medullary site of thymocyte apoptosis (Douek and Altmann, 2000). This was also

confirmed in our study by the detection of apoptotic cells in some Hassall's corpuscles.

There is substantial evidence that p27 is involved in cell cycle arrest and in the regulation of apoptosis (Lloyd et al., 1999). In the present study, the expression of p27 was rather alternative to the expression of Ki67, cyclin A and cyclin B1 proteins, in keeping with previous p27/Ki67 results on normal thymus (Sanchez-Beato et al., 1997). This is consistent with evidence that p27 concentration is reduced when cell-cycle progresses upon suitable mitogenic stimulation (Lloyd et al., 1999). The reduced p27 expression may permit sequential phosphorylation (inactivation) of Rb by the cyclin D/CDK4,6 and cyclinE-CDK2 complexes resulting in release of E2F from Rb in order to activate S-phase entry (Mulligan and Jacks, 1998) or may abrogate the association between cyclinE-CDK2 and p107-E2F complexes resulting in transcriptional activation of the mitotic cyclin A (Zerfass-Thome et al., 1997).

Besides its role in cell cycle, p27 has also been implicated in the regulation of apoptosis (Lloyd et al., 1999). Indeed, a recent study has provided evidence that thymocytes undergoing apoptosis degrade p27 and upregulate CDK2 activity, this process being regulated by p53, bax and bcl2 (Gil-Gomez et al., 1998). These findings are in keeping with our immunohistological observation that p27 expression increases from the cortex to the medulla in parallel with the decreased frequency of apoptotic cells, suggesting a role for p27 in protecting thymocytes from apoptosis.

A potential explanation may be related to recent in vitro results showing that p27 may protect mesangial cells and fibroblasts from apoptosis by costraining the CDK2 activity (Hiromura et al., 1999), which was shown to be essential for the apoptotic pathway used in negative thymic selection (Williams et al., 2000). Another explanation may be related to the Fas (CD95)-mediated cell death in thymus (Castro et al., 1996; Hersberger et al., 1998). There is evidence that Fas-mediated cell death occurs only during late G1 or G1/S phase transition whereas cells arrested in G1 are resistant to Fas-mediated apoptosis (Komada et al., 1995). Therefore, it could be suggested that p27 expression resulting in G1 arrest might render thymocytes resistant to Fas-mediated apoptosis. However, recent findings do not support this hypothesis since the CD95 pathway does not require CDK2 activation to induce cell death indicating that the biochemical pathway used in the CD95-mediated cell death plays no role in negative selection (Williams et al., 2000).

In summary, the present study reports the detailed immunotopographical distribution of proteins playing central roles in the regulation of the cell cycle and apoptosis in the human thymuses from various age-groups and provides in-situ evidence that the process of thymic involution may be related to the decrease of proliferation and increase of apoptosis.

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