Nucleolar organizer regions: their significance in protein synthesis and consequent release of factors that attract immature lymphocytes in different types of thymic epitheliocytes and in different stages of thymic development

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Summary

The thymus is a lymphoepithelial organ that has the central role in T-lymphocyte development. Unlike other lymphoid structures, where the supportive framework is chiefly collagenous reticular tissue, the thymus is permeated by a network of interconnected epithelial cells (thymic epitheliocytes) between which lodge lymphoid and other cells of the organ. There is much evidence that many distinctive functional roles are subserved by the thymic epitheliocytes such as, the differentiation of T lymphocytes, the production of soluble thymic factors or hormones, supportive functions, or their role in MHC restriction of T-cell immune responses.

Nucleolar organizer regions (NORs), which are important for regulating protein synthesis, were identified in 30 fetal thymuses in different stages of development (10th, 14th, 15th, 19th, 20th, 23rd, 31st, and 35th week), by means of a silver (Ag) staining technique (AgNOR).

The aim of our study was to estimate the AgNOR counts in the six different types of fetal thymic epitheliocytes, following suggestions that there may be a possible association between AgNOR values and consequent protein synthesis in the different types of these cells and in different stages of thymic development.

The results showed that: First Type I epitheliocytes (subcapsular-perivascular) of the cortex represent a higher number of AgNORs in comparison with the other cellular types, a difference that was observed every week of our study, and especially between the 10th and 15th week of development ($p < 0.01$). The increased number of AgNORs in Type I epitheliocytes reflect their intense protein synthesis, a fact that explains the increased secretion of factors, e.g. ß2-microglobulin, that release immature lymphocytes from the yolk sac and the liver. Second, gradual increase of the average AgNOR in all thymic epitheliocytes from the 10th till the 35th week, without any statistical variation. This increase might be due to the intense functional activity of the whole number of epitheliocytes that participate in the proliferation, differentiation and issue in the circulation of mature T lymphocytes, which takes place after the 17th week of development. The 17-week thymus appears fully differentiated, and after this time it produces the main type of thymocyte also present throughout life (designated TdT +).

Key words: Protein synthesis; immature lymphocytes; thymic epitheliocytes; thymic development.

Introduction

In the human thymus, the epitheliocytes have been divided morphologically into Types I-VI [1] and also characterized immunohistochemically [2]. There is considerable heterogeneity within these classes, and the two methods of analysis give slightly different results. Immunological reagents generally distinguish subcapsular, cortical and medullary epitheliocytes as well as Hassall's corpuscles. Some subcapsular and medullary cells share the same epitopes. According to the morphological classification, Type I epitheliocytes (subcapsular-perivascular) create the continuous monolayer around the perimeter of the thymus, extending along the septa to the corticomedullary boundary and forming an outer limit to the perivascular spaces. Like most other thymic epitheliocytes, Type I cells have MCCII-positive surfaces, apart from their capsule-facing aspects which are MHCII-negative. Type I cells secrete factors (e.g. ß2-microglobulin) which attract stem cells to the thymus [3], and thymic hormones [4]. Type II cells extend from the outer cortex towards the medulla forming a series of cells in contact with Types III and IV epithelial cells. All cortical epitheliocytes are closely opposed to thymocytes, sometimes apparently engulfing them (emperiopoleisis). Type V cells are a small subset of medullary epithelial cells which appear to be relatively unspecialized. Type VI cells are the commonest in the medulla, although several subsets may occur. Their forms range from spindle-shaped cells secreting thymic hormones to flattened cells forming Hassall's corpuscles.

The first lymphoid stem cells to enter the thymus in the embryo come from the yolk sac and liver during their haemopoietic phases, possibly, as in birds, being attracted by thymic chemotactic substances. During later periods it is probable that all thymic lymphocytes originate in the bone marrow, or at least have sojourned there, before passing through the bloodstream to the thymus.

Thymocytes undergo mitosis in all cortical zones as the clones of differentiating T cells mature, gradually moving deeper in the cortex. The appropriate conditions for the proliferation and differentiation of thymocytes appear to be produced by their close proximity to neighbouring epitheliocytes [5]. Although the nature of these interactions is not clear, it may involve the release from the

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epitheliocytes of soluble mitogenic and differentiation factors as well as induction of changes through intercellular contact. During this process, thymocytes differentiate along the T-cell line, acquiring the CD3 + marker and T-cell receptors, and also switching into different subclasses of T cells.

We searched for more objective methods with reference to approach the development and functional skills of the thymus. A new method for the estimation of cellular activity that is applied to a variety of neoplastic or hyperplastic lesions is the measuring of the nucleolar organizer regions. These regions correspond to large loops of transcribing DNA containing the ribosomal RNA genes (6). As rRNA molecules are the main sites of protein synthesis it follows that the number of NORs in each cell nucleus reflects cellular activity. Nucleolar organizer regions (NORs) can be readily identified in paraffin-embedded tissue by means of a silver (Ag) staining technique (AgNOR): they are visualised in the nuclei of cells as brown/black dots by virtue of the argyrophilia of NOR-associated proteins. These molecules were thought to include B23 and C23 subunits of RNA polymerase 1, and probably other phosphoproteins. The function of these molecules is still far from clear; they may partly reflect ploidy, though an increase in ploidy does not necessarily lead to an increase in AgNORs in human tumors.

For years cytogeneticists for the investigation of certain genetic disorders, notably that of chromosome 21 have used the AgNOR technique [7]. The recent modification of the original method [8], however, has become increasingly applied in histopathology research following suggestions that there may be a possible association between high AgNOR value and malignant transformation [8, 9, 10, 11, 12].

In this study we evaluated the AgNOR alterations in the six types of thymic epitheliocytes in order to compare and assess the possible differences in protein synthesis expressed by NORs in these cells, given that the thymus is not only a lymphopoietic but also an endocrine organ.

Our results are preliminary and to the best of our knowledge no relevant references exist in the literature to be compared with other investigators' findings.

In our work we considered only extra-nucleolar dots as AgNORs.

Materials and methods

Our cases comprised thymic tissue sections selected from 30 fetuses at different stages of development: Ten cases from the 10th till the 15th week, ten cases from the 19th till the 23rd week, and ten cases from the 31st till the 35th week of development. With regards to estimating the cellular and functional activity expressed by means of the numerical variation of NORs in the six types of thymic epitheliocytes, at the different stages of development, our material was divided into 12 groups, and a comparative study of our results was performed.

First group: comprised thymic epitheliocytes from the outer cortex (Type I, subcapsular) (10th, 14th, 15th week).

Second group: comprised thymic epitheliocytes from the outer cortex (Type I, perivascular) (10th, 14th, 15th week).

Third group: comprised thymic epitheliocytes from the outer and deep cortex (Type III and Type IV) (10th, 14th, 15th week).

Fourth group: comprised thymic epitheliocytes from the medulla (Type V and Type VI) (10th, 14th, 15th week).

Fifth group: comprised thymic epitheliocytes from the outer cortex (Type I, subcapsular) (19th, 20th and 23rd week).

Sixth group: comprised thymic epitheliocytes from the outer cortex (Type I, perivascular) (19th, 20th and 23rd week).

Seventh group: comprised thymic epitheliocytes from the outer and deep cortex (Type III and Type IV) (19th, 20th and 23rd week).

Eighth group: comprised thymic epitheliocytes from the medulla (Type V and Type VI) (19th, 20th and 23rd week).

Ninth group: comprised thymic epitheliocytes from the outer cortex (Type I, subcapsular) (31st and 35th week).

Tenth group: comprised thymic epitheliocytes from the outer cortex (Type I, perivascular) (31st and 35th week).

Eleventh group: comprised thymic epitheliocytes from the outer and deep cortex (Type III and Type IV) (31st and 35th week).

Twelfth: group comprised thymic epitheliocytes from the medulla (Type V and Type VI) (31st and 35th week).

Paraffin blocks, one from each case, were recut at 3 μm and sections were stained by the AgNOR silver colloid method as described by Smith and Crocker [13]. In brief, the sections were dewaxed in xylene, hydrated through graded ethanol, and washed with deionised water. They were exposed to freshly prepared AgNOR staining solution: one volume of a 2% solution of gelatin in 1% formic acid mixed with two volumes 50% aqueous silver nitrate solution. The reaction was performed at room temperature in the dark for 30 min. The silver colloid was then washed off with running deionised water and sections were dehydrated through alcohol to xylene and mounted in synthetic medium.

AgNORs were measured in 100 cells as suggested by Howat's group [14]. In this procedure only discrete, easily discernible, black dots were enumerated. Cells were examined using a X100 oil immersion objective and microscopical fields were randomly selected. Three observers performed AgNOR counts and no significant divergences were noted. The analysis of the results was performed by the Student's unpaired t-test.

Results

AgNORs were observed in almost the majority of the nuclei of the thymic epitheliocytes in all fetal thymic tissues examined at different weeks of development (Figure 1).

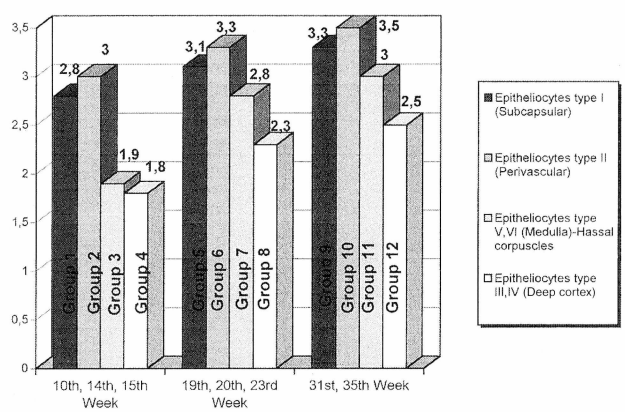

Diagram 1.

1st group:	AgNOR mean value in the thymic epitheliocytes from the outer cortex (Type I, subcapsular) was 2.8 ± 0.6 (1.7 - 3.5) (at the 10th, 14th, 15th week).
2nd group:	AgNOR mean value in the thymic epitheliocytes from the outer cortex (Type I, perivascular) was 3.0 ± 0.7 (2.1 - 4.2) (at the 10th, 14th, 15th week).
3rd group:	AgNOR mean value in the thymic epitheliocytes from the outer and deep cortex (Type III and Type IV) was 1.9 ± 0.6 (1.2 - 2.7) (at the 10th, 14th, 15th week).
4th group:	AgNOR mean value in the thymic epitheliocytes from the medulla (Type V and Type VI) was 1.8 ± 0.7 (0.9 - 2.7) (at the 10th, 14th, 15th week).
5th group:	AgNOR mean value in the thymic epitheliocytes from the outer cortex (Type I, subcapsular) was 3.1 ± 0.8 (2.1 - 4.3) (at the 19th, 20th and 23rd week).
6th group:	AgNOR mean value in the thymic epitheliocytes from the outer cortex (Type I, perivascular) was 3.3 ± 0.7 (2.2 - 4.5) (at the 19th, 20th and 23rd week).
7th group:	AgNOR mean value in the thymic epitheliocytes from the outer and deep cortex (Type III and Type IV) was 2.8 ± 0.5 (1.9 - 3.6) (at the 19th, 20th and 23rd week).
8th group:	AgNOR 1 mean value in the thymic epitheliocytes from the medulla (Type V and Type VI) was 2.3 ± 0.6 (1.5 - 3.5) (at the 19th, 20th and 23rd week).
9th group:	AgNOR mean value in the thymic epitheliocytes from the outer cortex (Type I, subcapsular) was 3.3 ± 0.6 (2.5 - 4.6) (at the 31st and 35th week).
10th group:	AgNOR mean value in the thymic epitheliocytes from the outer cortex (Type I, perivascular) was 3.5 ± 0.7 (2.6 - 4.7) (at the 31st and 35th week).
11th group:	AgNOR mean value in the thymic epitheliocytes from the outer and deep cortex (Type III and Type IV) was 3.0 ± 0.6 (2.1 - 3.9) (at the 31st and 35th week).
12th group:	AgNOR mean value in the thymic epitheliocytes from the medulla (Type V and Type VI) was 2.5 ± 0.6 (1.9 - 3.6) (at the 31st and 35th week).

A comparative study between the groups was performed and the statistical analysis showed:

1. No statistically significant difference in the NOR mean value between thymic epitheliocytes from the outer cortex (Type I, subcapsular) (groups 1, 5, and 9) and the thymic epitheliocytes from the outer cortex (Type I, perivascular) (groups 2, 6, and 10) in all weeks of development ($p > 0.1$).

2. A statistically very significant difference in the NOR mean value between thymic epitheliocytes from the outer cortex (Type I, subcapsular) (groups 1, 5, and 9) and thymic epitheliocytes from the outer and deep cortex (Type III and Type IV) (groups 3, 7, and 11) in all weeks of development ($p < 0.01$).

3. A statistically significant difference in the NOR mean value between thymic epitheliocytes from the outer cortex (Type I, subcapsular) (groups 1, 5, and 9) and the thymic epitheliocytes from the medulla (Type V and Type VI) (groups 4, 8, and 12) in all weeks of development ($p < 0.01$).

4. A statistically significant difference in the NOR mean value between thymic epitheliocytes from the outer cortex (Type I, perivascular) (groups 2, 6, and 10) and thymic epitheliocytes from the outer and deep cortex (Type III and Type IV) (groups 3, 7, and 11) in all weeks of development ($p < 0.01$).

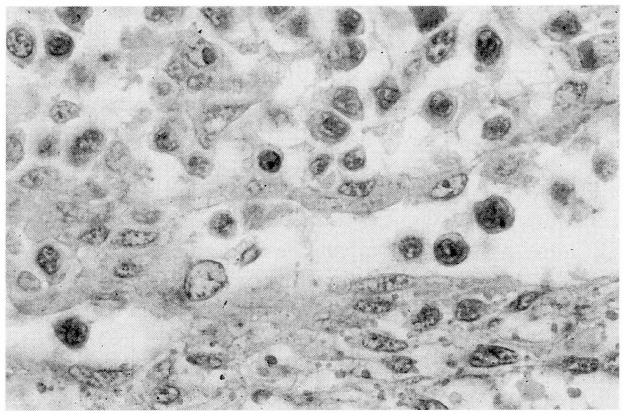

Figure 1. — AgNORs are observed in almost the majority of the nuclei of the thymic epitheliocytes in all fetal thymic tissues examined at different weeks of development (AgNOR stain x250).

5. A statistically significant difference in the NOR mean value between thymic epitheliocytes from the outer cortex (Type I, perivascular) (groups 2, 6, and 10) and the thymic epitheliocytes from the medulla (Type V and Type VI) (groups 4, 8, and 12) in all weeks of development ($p < 0.01$).

6. No statistically significant difference in the NOR mean value between thymic epitheliocytes from the outer and deep cortex (Type III and Type IV) (groups 3, 7, and 11) and the thymic epitheliocytes from the medulla (Type V and Type VI) (groups 4, 8, and 12) in all weeks of development ($p > 0.1$).

Discussion

The thymus is a lymphopoietic organ responsible for the development and maturation of any other peripheral lymphoid organs. Two major functions are served by the thymus: 1) The production of thymic hormones known as thymosins, thymulin, and thymopoietin which trigger T-cell differentiation, and 2) The supplying of the peripheral lymphoid organs with lymphocytes.

The well-characterized thymic hormones are principally thymulin, the thymosins, thymopentin and thymic humoral factor. Thymulin, originally called 'Facteur Thymique Serique' (FTS) relies on zinc for its biological activity [15]. The thymosins are a large family isolated from thymosin fraction 5 [16]. The precursor of thymosin 1a, prothymosin, is found in highest quantities in the thymus but is also secreted elsewhere [17] as is parathymosin. Thymosin b1 is ubiquitin [18], and thymosins b4 and b10 have recently been shown to be sequestering components of connective tissue [19]. Thymopoietin, although originally studied for its neuromuscular effects, has its biological and immunological activity in residues 32-36 (thymopentin or TP5) [20]. Thymic humoral factor (THF)

had also been sequenced [21] and no homology has been found between any of the thymic hormones.

The peptide hormones of the thymus have a range of immunomodulatory effects on lymphocyte maturation within the thymus and in the periphery [15, 22, 23]. Most will induce markers of early differentiation on lymphoid cells lacking such markers, and enhance various T-cell functions. The injection of most thymic hormones restores immunological competence to neonatally thymectomized mice, modulates surface epitopes in patients with immune deficiencies and improves immunocompetence in man and animals.

There are many other soluble factors in the thymic microenvironment, but cytokines have been shown (usually by in vitro methods) to be important singly or synergistically in thymocyte development. Their actions are very complex, and not yet fully understood. Interleukins IL-1, IL-2, IL-4 and IL-6 are secreted by thymocytes (as well as other cell types), and IL-1, IL-3, IL-4, IL-6 and IL-7 by the thymic epithelium. Cells bearing receptors for all of these cytokines, as well as for tumour necrosis factor a (TNFa), and colony stimulating factors for granulocytes and macrophages (GM-CSF, M-CSF or CSF-1) and g-IFN, occur in the thymus.

All major hormones released from endocrine glands can influence thymic function and/or structure, and thymic factors often affect other endocrine organs. Many of these effects are mediated through the thymic microenvironment. Thymic epithelial cells have receptors for all of the sex steroid hormones [24, 25], including a unique oestrogen receptor, corticosteroid receptors, nuclear receptors for triiodothyronine [26] and low-affinity luteinizing hormone releasing hormone (LHRH) receptors. Receptors on thymocytes have been identified for growth hormone [27], corticosteroids (lower numbers than on epithelial cells), oxytocin [28], and oestrogen (fewer than on epithelial cells) [29].

Lymphocyte production in the thymus is gradually increased till the twelfth year of age; then it slows down but it is continued till senile age.

Nowadays we believe that interactions between thymocytes and thymic microenvironment elements, thymic hormones (thymosins, thymolin and thymopoietin), interleukin 1 and 2 are responsible for the differentiation and maturation of T- lymphocytes.

Thymic epitheliocytes release hormones such as thymosins, thymolin and thymopoietin which control the proliferation, differentiation and maturation of T-lymphocytes in the thymus, but also regulate the function and interactions of T-cells in the peripheral lymphoid organs.

Our results show that 1) Subcapsular and perivascular epitheliocytes of the cortex demonstrate higher AgNOR counts than all other types of cells, during all the weeks in this study, and especially between the 10^{th} and 15^{th} week ($p < 0.01$). Increased NORs in these cells correspond to intense protein synthesis, a fact that explains the increased secretion of all factors that attract immature lymphocytes (e.g. ß-microglobulin) from the yolk sac and the liver. 2) The results also show a gradual increase of the mean AgNOR count in all types of epitheliocytes from the 10th till the 35th week with no statistical variation. This increase could be explained by the intense functional skills of all epitheliocytes participating in the proliferation, differentiation and release in the circulation of mature T-lymphocytes after the 17^{th} week of development.

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