D. Tamiolakis, MD, Director, Department of Cytology, General Hospital of Alexandroupolis, J. Venizelos, PhD, Associate Director, Department of Pathology, Ippokration Hospital of Salonica, A. Kotini, PhD, Lecturer, Department of Medical Physics, Democritus University of Thrace, D. Karamanidis, PhD, Associate Director, Department of Obstetrics and Gynecology, General Hospital of Alexandroupolis, P. Boglou, Department of Histology - Embryology, Democritus University of Thrace, Greece, and N. Papadopoulos, PhD, Associate Professor, Director,Department of Histology - Embryology, Democritus University of Thrace, Greece

Request for reprints to: Prof. N. Papadopoulos, Department of Histology-Embryology, Democritus University of Thrace, Dragana, 68 100 Alexandroupolis, Greece

A STROMAL MYOID CELL LINE PROVOKES THYMIC ERYTHROPOIESIS BETWEEN 16TH TO 20TH WEEKS OF INTRAUTERINE LIFE

D. TAMIOLAKIS, J. VENIZELOS, A. KOTINI, D. KARAMANIDIS, P. BOGLOU and N. PAPADOPOULOS

ABSTRACT

Background: The thymus provides an optimal cellular and humoral microenvironment for cell line committed differentiation of haematopoietic stem cells. The immigration process requires the secretion of at least one peptide called thymotaxine by cells of the reticulo-epithelial (RE) network of the thymic stromal cellular microenvironment. The thymic RE cells are functionally specialised based on their intrathymic location and this differentiation is modulated by various interaction signals of differentiating thymocytes and other non lymphatic haematopoietic stem cells.

Objectives: To study the role of another cell line in fetal thymic haematopoietic proliferation and differentiation in different stages of development: the stromal myoid cells.

Design: Fifteen cases of fetal thymic specimens (4th to 8th weeks: five cases 16th to 20th weeks: five cases and 28th to 32nd weeks: five cases respectively) were studied. Tissue paraffin samples were stained immunohistochemically using (i) a monoclonal antibody recognising alpha-smooth muscle actin, a contractile microfilament expressed exclusively by smooth muscle cells, myofibroblasts and related cells, (ii) a monoclonal antibody glycophorin C recognising the erythropoietic cells.

Setting: Histology - Embryology Department of Democritus University of Thrace (Alexandroupolis) over ten year period (1991-2001).

Results: The number of alpha-smooth muscle actin - positive cells significantly increased during the late second and third trimester of gestation. In the above period a relevant increase in the number of glycophorin C positive cells were observed.

Conclusion: Our data suggest that a myoid cell line is involved in the formation of an appropriate microenvironment for homing and proliferation of erythropoietic cells.

INTRODUCTION

Myoid cells (MCs) constitute a normal component of the thymic connective tissue stroma. These cells are situated mainly in the medulla and at the corticomedullar junction. They are large, rounded cells, with a central nucleus surrounded by irregularly arranged bundles of myofilaments. In lower vertebrates, where myoid cells are often more numerous, these cells are joined to neighbouring medullary epithelial cells by desmosomes. Their functions are unknown, although it has been suggested that their contractions might aid the movement of lymphoid cells across or out of the gland.

Myoid cells share some characteristics with thymic epithelial cells(1), and it has been suggested that myoid cells may derive from myoepithelial cells within the thymus(2). Other reports have suggested that myoid cells come from

pluripotent stem cells(3), or from endodermal reticular cells. It has also been postulated that they are of extrathymic origin, arising during embryogenesis from muscle precursor cells of the surrounding mesoderm(1). Experiments with chick/quail chimeras do not support the notion that myoid cells arise from transdifferentiation of thymic epithelial cells but are rather of neuroectodermal origin(4). Thymic myoid cells express several muscle-specific proteins including troponin T, desmin(5), and the acetylcholine receptor (AChR)(5)(6). They have therefore the antigenic characteristics of the skeletal muscle cells within the thymus(7). Their biological role is unclear, but their involvement in human myasthenia gravis (MG) has been suggested(3). Van de velde and Friedman reported that myoid cells were present in thymus and in thymoma from both young and adult patients with MG(8).

MATERIALS AND METHODS

Materials: Fifteen cases of thymic foetal specimens in different stages of development obtained after pregnancy failure due to chorioamnionitis (five samples), to implantation of the foetus in the region of the internal os resulting in placenta praevia (six samples), and to endometriosisadenomyosis (four samples).

Selection Criteria: All cases were chosen from specific time of development (4th to 8th weeks: five cases, 16th to 20th weeks: five cases and 28th to 32nd weeks: five cases respectively). The study was executed in harmony with the guidelines for the analysis of foetal cells, tissues and approved by the Ethics Committee of the General Hospital of Alexandroupolis.

Study Design: Tissue samples were processed for paraffin section immunophenotyping and stained using the monoclonal antibodies against alpha-smooth muscle actin (alpha-SMA) and glycophorin C by DAKO.

Methods: The sections were examined independently by two observers and positive cellular staining for alpha-smooth muscle actin and glycophorin C antibodies were manifested as fine yellow cytoplasmic granularity and/or surface membrane expression. They were examined with a X 40 objective and the distribution of the above antigens within the cell was recorded. Every stained cell was scored as positive regardless of staining intensity. To count the number of cells with alpha-smooth muscle actin and glycophorin C stainings, a 10 by 10 square calibrated grid was inserted into the eyepiece of an Olympus BX40 binocular microscope. Five-to-ten fields were examined for each section, and at least 1000 cells were scored, depending on cellularity. The percentage of positive cells were recorded as the relevant indices.

$$\text{alpha-smooth muscle actin index} = \frac{\text{no. of positive cells}}{\text{no. of total (positive+negative cells)}}$$

$$\text{Glycophorin C index} = \frac{\text{no. of positive cells}}{\text{no. of total (positive+negative cells)}}$$

The indices ranged from 0-100%, with a mean of 18%. The mean index was evaluated in three ranges: low index (under 18%), grade I; moderate index (from 18 to 50%), grade II; and high index (from 51 to 100%), grade III. The statistical analysis was obtained using the t-test. The mean values were expressed as average $\pm$SD.

RESULTS

Medullary stromal cells expressed alpha-smooth muscle actin in 7 of 15 fetuse-cases during the 4th to 8th weeks (46.66%) (32.75$\pm$1.91 cells/mm^2), in all 15 cases during the 16th to 20th weeks (100%) (48.61$\pm$4.43 cells/mm^2) and in 8 of 15 cases during the 28th to 32nd weeks (53.33%) (29.37$\pm$3.29 cells/mm^2). Erythropoietic cells, expressed glycophorin C in 10 of 15 fetuse-cases during the 4th to 8th weeks (66.66%) (55.41$\pm$3.12 cells/mm^2), in all 15 cases during the 16th to 20th weeks (100%) (67.93$\pm$4.35 cells/mm^2), and in 13 of 15 cases during the 28th to 32nd weeks (86.66%) (101.46$\pm$6.76 cells/mm^2).

Figure 1

Immunohistochemical control for alpha-smooth muscle actin showing a strong reactivity with the stromal myoid cells of the thymic medulla. X250

Figure 2

Immunohistochemical control for glycophorin C showing a strong reactivity with the progenitor haematopoietic stem cells, progenitor stromal cells and endothelial cells of the vessels of the thymic medulla. X250

Table 1

Expression of alpha-SMA and glycophorin C in fetal thymic medulla

	4-8 weeks	16-20 weeks	Statistical analysis
a-SMA (+)cells/mm^2	32.72 ± 1.91	48.61 ± 1.43	$p<0.0001$
Glycophorin C (+) cells/mm^2	55.41 ± 3.12	67.93 ± 4.35	$p<0.0001$
	16-20 weeks	28-32 weeks	Statistical analysis
a-SMA (+)cells/mm^2	48.61 ± 1.43	29.37 ± 3.29	$p<0.0001$
Glycophorin C (+) cells/mm^2	67.93 ± 4.35	101.46 ± 6.76	$p<0.0001$

Our results demonstrated a statistically significant difference in the 16th to 20th weeks of gestation concerning the expression of alpha-smooth muscle actin in the connective tissue stroma of the thymic medulla over the equivalent expression of the protein in the 4th to 8th weeks (p<0.0001, t-test) and 28th to 32nd weeks (p<0.0001, t-test). Similar changes in the above period were found concerning the expression of glycophorin C over the 4th to 8th weeks (p<0.0001, t-test) and 28th to 32nd weeks (p<0.0001), suggesting a direct involvement of alpha-smooth muscle actin in the backing up of erythropoietic activity.

Out of seven fetuse-cases with positive alpha-smooth muscle actin expression during the 4th to 8th weeks, five were scored as grade I, and II as grade III. Out of 15 fetuse-cases with positive, alpha smooth muscle actin expression during the 16th to 20th weeks, four were scored as grade one, nine as grade II, and two as grade III (Figure 1). Out of eight fetuse-cases with positive alpha-smooth muscle actin expression during the 28th to 32nd weeks, four were scored as grade one, two as grade II, and two as grade III.

Out of ten fetuse-cases with positive glycophorin C expression during the 4th to 8th weeks, five were scored as grade one, three as grade II and two as grade III. Out of 15 fetuse-cases with positive. Glycophorin C expression during the 16th to 20th weeks, three were scored as grade I, seven as grade II, and five as grade III (Figure 2). Out of 13 fetuse-cases with positive glycophorin C expression during the 28th to 32nd weeks, two were scored as grade I, eight as grade II, and three as grade III.

DISCUSSION

Thymic stromal cells are thought to play a critical role in the proliferation, differentiation and selection of precursor cells in the T-cell lineage, but the precise mechanisms by which these events occur, and the particular contribution of individual thymic stromal components, are largely unknown. Most cells of the thymic stroma are of epithelial origin. Human thymic epithelial cells have been shown to produce numerous cytokines including IL-1, IL-6, granulocyte colony-stimulating factor (G-CSF), and macrophage CSF (M-CSF), that are important in various stages of thymocyte differentiation(9).

Haemopoietic cells are present in foetal life, when the thymus makes an important contribution to the formation of erythrocytes and leukocytes. Later haemopoietic cells are often present, possibly as a result of the reactivation of persistent foetal stem cells. Normoblasts have been found in the thymuses of many adult open-heart surgery patients, and immature eosinophils, neutrophils and mast cells have also been observed. Where present in adults, the erythropoietic foci are mainly in the subcapsular and outer cortex.

The role of myoid cells has been explored by co-culturing the myoid cells with thymocytes. Interestingly, the myoid cell line appears to protect thymic cells from spontaneous apoptosis, while the human thymic epithelial cell line has no effect. The mechanism of the protective effect needs further investigation. It could be mediated by soluble factors or by direct cell contacts. The presence of the adhesion molecule LFA-3 (CD58) on myoid immortalised thymic cells (MITC)(10) makes possible interactions between myoid cells and most thymocytes that constitutively express CD2. Indeed LFA-3 is important in the interaction with thymocytes at both immature and mature stages of development(11).

Human thymic myoid cells can be immortalised from thymic explant cultures. These cells express both the foetal and adult forms of muscle acetylcholine receptor (AChR) at the mRNA level. A Subunit AChR protein expression is detectable in MITC line by flow cytometry. MITC line express a functional AChR, as shown by patch-clamp analysis. AChR expression on MITC line is down-modulated by myasthenia gravis (MG) sera(15), as on TE671 rhabdomyosarcoma cells, making MITC line an interesting tool for AChR antigenic modulation experiments. MITC line produces high levels of TNF-a and IL-8 and protects thymocytes from apoptosis, indicating that thymic myoid cells could play a role in thymocyte differentiation(10).

The following experimental arguments support the myoid nature of MITC line:
(i). In the postnatal thymus, thymic myoid cells express several striated-muscle-specific proteins, including myosin(12), desmin and troponin T (5). Accordingly, Wakkach *et al.*(10) found that MITC line was troponin T-positive and desmin-positive, but keratin-negative. In addition, the MITC line has similar phenotype characteristics as myoid cells *ex vivo,* according to AChR, MHC class

I and class ll, LFA-3, and ICAM-1 antigens.

(ii). Wakkach's *et al.*(10) data clearly indicate the presence of MyoD transcripts in MITC cells. MyoD is expressed only in skeletal muscle, and activates myogenesis by directly binding to the control regions of muscle-specific genes(13). Pluripotential stem cells in the thymus might express such a gene and develop into immature skeletal muscle cells in certain conditions. This is supported by the fact that cell types as different as osteocytes and chondrocytes can differentiate from thymus cells in appropriate conditions *in vitro*(14).

(iii). Human MITC line expressed AChR in both adult and foetal forms(10). In addition, anti-AChR auto antibodies induced a loss of AChR on MITC cells *in vitro,* similarly to TE671 cells; this mechanism was also observed using the anti-MlR monoclonal antibody MAb 35. These results indicate that AChR present on these cells is recognised by antibodies found in MG sera.

Some of MG patients' antibodies may show a marked preference for adult or foetal AChR, and some patients (up to 7%) are negative in diagnostic assays using only foetal AChR. That could explain why 10 to 15% of patients with clinical MG have very low titers of antibodies (0.2 to 2 nmol/L) (15). To enhance the sensitivity of the diagnostic assay for low-titer sera, Beeson *et al.* (16) used TE671 cells transfected with the epsilon subunit of AChR as a source of AChR antigen. Thus a mix of AChR extracts from TE671-e and TE671 cells, in which adult and foetal AChR are present, was as sensitive as AChR from amputated leg muscle in MG diagnostic assay. Wakkach's *et al.*(10) data show that MITC line expresses constitutively the adult and the foetal forms of the AChR, thus MITC line might be an appropriate source of AChR for titrating anti-AChR antibodies in MG sera, as well as for studies of their functional effects. Expression of major histocompatibility complex (MHC) molecules by components of the thymic microenvironments required for normal T cell development(17) and has been implicated in the selection of the emerging T cell repertoire.

In our series: (i) The comparative study of the quantitative percentage of alpha-smooth muscle actin expression at. 1st, 2nd and 3rd trimester of gestation showed a statistically significant difference in the number of stromal myoid cells in the thymic medulla during the second trimester over the first ($p<0.0001$, t-test) and third trimesters ($p<0.0001$, t-test).

(ii) The comparative study of the quantitative percentage of glycophorin C for the identification of erythropoietic cells, at 1st, 2nd and 3rd trimester of gestation showed a statistically significant difference in the number of the relevant cells of the thymic parenchyme during the second trimester over the first (p 0.0001 , t-test) and third trimesters ($p<0.0001$, t-test).

Taken together, our data indicate that stromal cells expressing alpha-smooth muscle actin upregulate thymic haematopoiesis, providing a suitable background for the proliferation and differentiation of erythropoietic cells in the fetal thymus. Our conclusion may be not a safe one due to the limited number of cases examined, but it is certainly intriguing for further investigation.

REFERENCES

1. Puchtler, H., Meloan, S. N., Branch, B. W., Gropp, S. Myoepithelial cells in human thymus: staining, polarisation and fluorescence microscopic studies. *Histochemistry* 1975; **45:**163-176.

2. Dardenne, M., Savino, W. and Bach, J. F. Thymomatous epithelial cells and skeletal muscle share a common epitope defined by a monoclonal antibody. *Amer. J. Pathol.* 1987; **126:**194-198.

3. Wekerle, H., Hohlfeld, R., Ketelsen, U. P., Kalden, J. R. and Kalies, I. Thymic myogenesis, T-Lymphocytes and the pathogenesis of myasthenia gravis. Myasthenia Gravis. Edited by Drachman, D. B. *Ann. NY. Acad. Sci.* 1981; pp 455-476

4. Nakamura, H. and Ayer Le Lievre, C. Neural crest and thymic myoid cells. *Curr. Trop. Dev. Biol.* 1986; **20:**111-115.

5. Schluep, M., Willcox, N., Vincent, A., Dhoot, G. K. and Newsom, Davis J. Acetylcholine receptors in human thymic myoid cells *in situ:* an immunohistological study. *Ann. Neurol.* 1987,;**22:** 212-222.

6. Kao, I. and Drachman, D. B. Thymic muscle cells bear acetylcholine receptors: possible relation to myasthenia gravis. *Science.* 1977; **195:**74-75.

7. Seifert, R. and Christ, B. On the differentiation and origin of myoid cells in the avian thymus. *Anat Embryol.* (Berl) 1990; **181:**287-298.

8. Van de Velde, R. L., Friedman, N. B. Thymic myoid cells and myasthenia gravis. *Am. J. Pathol.* 1970; **59:**347-368.

9. Meilin, A. Shoham, J. and Sharabi, Y. Analysis of thymic cell subpopulation grown *in vitro* on extracellular in defined medium. IV. Cytokines secreted by human thymic epithelial cells it, culture and their activities on murine thymocytes and bone marrow cells. *Immunology.* 1992; **77:** 208-213.

10. Wakkach, A., Poea, S., Chastre, E., *et al.* Establishment of a human thymic myoid cell line. Phenotypic and functional characteristics. *Amer. J. Pathol.* 1999, **155:**1229-1249.

11. Singer, K. M., Denning, S. M., Whichard, L. P. and Haynes, B. F. Thymocyte LFA-1, and thymic epithelial cell ICAM-1 molecules mediate binding of active human thymocytes to thymic epithelial cells. *J. Immunol.* 1990, **144:**2931-2939.

12. Drenckhahn, D., von Gaudecker, B., Muller Hermelink, H. K., *et al.* Myosin and actin containing cells in the human postnatal thymus: ultrastructural and immunohistochemical findings in normal thymus and in myasthenia gravis. *Virchows Arch. B Cell Pathol.* 1979, **32:**33-45.

13. Lassar, A. B., Buskin, J. N., Lockshon, D., *et al.* MyoD is a sequence-specific DNA binding protein requiring a region of mychomology to bind to the muscle creatine kinase enhancer. *Cell.* 1989; **58:**823-831.

14. Friedenstein, A. J. and Lalykina, K. S. Thymus cells are inducible to osteogenesis. *Eur. J. Immunol.* 1972, **2:** 602-603.

15. Vincent, A. and Newsom, D., J. Acetylcholine receptor antibody characteristics in myasthenia gravis. III. Patients with low anti-AChR antibody levels. *Clin. Exp. Immunol.* 1985, **60:**631-636.

16. Beeson, D., Jacobson, L., Newsom, D., J., and Vincent, A. A transfected human muscle cell line expressing the adult subtype of the human muscle acetylcholine receptor for diagnostic assays in myasthenia gravis. *Neurology* 1996, **47:**1552-1555.

17. Kruisbeek, A. M., Mond, J. J., Fowlkes, B. J., *et al.* Absence of the Lyt-2, L3T4+ lineage of T cells in mice treated neonatally with anti-l-A correlates with absence of intrathymic I-A-bearing antigen-presenting cell function. *J. Exp. Med.* 1985, **161:**1029-1047.