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A comparative immunohistochemical investigation of the consequences of chorioamnionitis on the developing human fetal spleen

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Summary

Introduction: The objective of this study was to determine the effects of chorioamnionitis on the extracellular matrix (ECM) structural glycoproteins of the developing human fetal spleen, and their influence on the haematopoiesis and spleen immune system compared to controls. *Materials and Methods:* After elective induced pregnancy termination due to chorioamnionitis or voluntary abortion, paraffin-embedded specimens from the spleen and respective fetal membranes of 90 fetuses were investigated by immunohistochemistry for presence of ECM structural glycoproteins, haematopoietic, and lymphoid cells. Conventional histological examination of the relative fetal membranes was performed. *Results:* The present results showed no quantitative variations in the expression of the ECM glycoproteins and haematopoietic lineages of the fetal spleen parenchyma at the end of first trimester (in both groups). At the second and third trimesters, acute chorioamnionitis showed a decreased number of the aforementioned proteins, with an increase of granulopoiesis and CD34 progenitor/stem haematopoietic cells. The immune system of the spleen during the third trimester demonstrated a decrease of both B and T lymphocytes, in comparison with controls. *Conclusions:* These results suggest that toxins and cytokines generated during chorioamnionitis, seem to influence ECM structural glycoproteins synthesis and release in fetal splenic parenchyma by reducing them, and probably cause further disorders of haematopoiesis and lymphopoiesis.

Key words: Fetal spleen; Chorioamnionitis; Structural glycoproteins; Haematopoiesis; Lymphopoiesis.

Introduction

The spleen is an organ derived directly from mesoderm and during embryogenesis is recognized from the fifth week of gestation, whereas blood vessels, red and white pulp, appear after the ninth week [1, 2]. It is well established that haematopoiesis occurs first in the yolk sac, then in the liver, spleen and other fetal organs, and finally in the bone marrow [3-7]. Hematopoiesis takes place in the human fetal spleen during the second trimester of gestation and the extracellular matrix meshwork of this organ has long been recognized as a major anatomical component of the spleen microenvironment. The development of the immune system begins from the end of the second semester when B-and T-cell areas can be recognized and this development continues after birth [8-11]. The process of phagocytosis is testified at the 12th week of gestation [12]. Congenital developmental changes of the spleen include accessory spleens [13], polysplenia [14], and asplenia [15].

Several proteins mediate interaction between cells and extracellular matrix and interact with specific receptors on the cell surface [16]. The distribution of such proteins varies between different tissues. The best characterization of these proteins are tenascin-C, laminin, and entactin (nidogen) [17]. Tenascins (Tn) are a family consisting of five members, Tenascins -C, R, X, Y, and W. The most extensively investigated regarding growth and regeneration is tenascin-C [18]. Tenascins are characterized by a spatially and temporally restricted tissue distribution during embryonic development, inflammatory processes,wound healing, and neoplasmatic invasion. During embryonic development, expression of tenascins at sites of epithelial-mesenchymal interactions is one of the most distinct characteristics of these proteins [19, 20]. In the lymphoid tissue, tenascin is able to exert important immunomodulatory activities on T and B-lymphocytes as well as on the macrophages. [21]. Laminin, is a sulfated glycoprotein, constituting a major component of basement membranes and synthesized at high levels during tissue growth and development [22]. It is produced by most epithelial and endothelial cells, and is a cross-shaped molecule with binding sites for specific cell receptors-integrins, heparin sulfate, type IV collagen, and entactin [23-25]. The multiple binding ligands for laminin make it a major extracellular link molecule between cells and extracellular matrix. There are

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several forms of laminin specific to different tissues [26]. The entactin-nidogen family, representing multivalent matrix binding proteins, consists in mammals of two members, nidogen-1 (entactin) and nidogen-2. They are considered as classical linkers joining laminin and collagen IV networks in basement membranes [27, 28]. Nidogen-1 (entactin) is an integral and ubiquitous component in nearly all basement membranes, but at times also contributes to the extracellular matrix of mesenchymal tissues [29, 30]. The regulation of haematopoiesis and lymphopoiesis seems to be an intricate procedure, in a general aspect. This procedure is the amazing result of many individual processes, such as interactions between cells, and between cell and extracellular matrix's components. Furthermore, cytokines, determined growth factors and intrinsic molecules, which act as modulators, seem to play an important role in haematopoietic development [31].

Since 1975 [32] there have been many case reports in the literature dealing with the consequences of chorioamnionitis. Chorioamnionitis is responsible for up to one-third of spontaneous abortions during the second trimester of gestation and is associated with an increased risk for injury in many organs of the developing fetus, as well as with an increased risk of early-onset sepsis [33-37]. The inflammatory infiltrate in the fetal membranes and the umbilical cord consists of polymorphonuclear leukocytes with a migratory trend and attitude toward the amnionitic cavity. The fetus eventually is expelled because uterine contractions can no longer be suppressed after a certain stage of the disease [38-40]. Little is known in the developing human fetal spleen about the behavior, distribution, and expression of the ECM structural glycoproteins (tenascin-C, laminin, and entactin), when they are under the effects of chorioamnionitis. The impact of histologically confirmed changes of acute chorioamnionitis on the fetal spleen has not yet been investigated.

The purpose of this retrospective study was to provide in the human fetal splenic parenchyma, a quantitative overview of ECM molecules (tenascin-C, laminin, and entactin) and activities of haematopoiesis and lymphopoiesis in fetuses featuring changes of acute chorioamnionitis in the fetal membranes comparing the present results with those cases without evidence of chorioamnionitis.

Materials and Methods

Tissue specimens

Paraffin-embedded specimens of 90 post-mortem human fetal spleens with their respective fetal membranes resulting from elective abortions (clinical signs of chorioamnionitis), were obtained from the archives (2001-2014) of the Department of Histology–Embryology, Medical Faculty of Democritus University of Thrace, Alexandroupolis, Greece. Thirty fetuses corresponded to 10^{th} to 12^{th} gestational weeks (10×10^{th}, 10×11^{th}, 10×12^{th} week of gestation), ten of them featuring fetal membranes with mild changes of acute chorioamnionitis, ten with moderate changes, and ten with severe changes. Thirty fetuses corresponded to 13^{th} to 24^{th} gestational weeks (5×13^{th}, 5×14^{th} 5×16^{th}, 5×18^{th}, 5×20^{th}, and 5×24^{th} week of gestation), featuring ten with mild changes of acute chorioamnionitis, ten with moderate changes, and ten with severe changes, and further 30 fetuses corresponded to 25^{th} to 32^{th} gestational weeks (5×25^{th}, 5×28^{th}, 10×30^{th}, and 10×32^{nd} week of gestation), featuring ten with mild changes of acute chorioamnionitis, ten with moderate changes, and ten with severe changes. Specimens of spleen with their respective fetal membranes from 30 cases of voluntary abortion due to endometriosis-adenomyosis and implantation of the fetus in the region of the internal os resulting in placenta previa and leiomyoma, were examined as well and used as control subjects and comparative means in this study. Microscopic examination of the control subjects did no show evidence of chorioamnionitis. Of them, ten fetuses corresponded to 10^{th} to 12^{th} gestational weeks (5×10^{th}, 5×12^{th} week of gestation), ten to 16^{th} to 24^{th} gestational weeks (2×16^{th}, 2×18^{th}, 2×20^{th}, 2×22^{th}, and 2×24^{th} week of gestation) and further ten in the 25^{th} to 32^{th} gestational weeks (2×25^{th}, 2×28^{th}, 2×30^{th}, and 4×32^{nd} week of gestation). Fetuses belonging to twin and multiple pregnancies were excluded from the study. Gestational age was estimated using developmental criteria. The study was executed in harmony with the guidelines for the analysis of fetal cells and tissues and was approved by the ethics committees of the Democritus University of Thrace.

Methods

Fetal spleens with their respective placentas were cut as thick as five mm, then fixed in 10% neutral buffered formaldehyde at 4°C for 24 hours and processed for routine paraffin embedding. Paraffin blocks were available in all cases. Four-micrometer-thick paraffin sections were cut from blocks containing sections from the spleen with their respective placentas. For conventional histological analysis, the sections were stained with haematoxylin and eosin (H&E). The histological features of all cases were reassessed on the basis of sections stained with H&E. Representative paraffin blocks were available for immunohistochemical evaluation.

Immunohistochemistry

A panel of antibodies was applied using immunohistochemistry. A streptavidin–biotin peroxidase complex method was employed for the immunohistochemical steps. Particularly, to detect antigens present in extracellular matrix of the mesenchymal tissue of the fetal spleens were included the following antibodies: anti-TNC monoclonal rabbit antibody for detection of tenascin-C in a dilution of 1:200, anti-Laminin polyclonal rabbit antibody for detection of laminin a dilution of 1:50, anti-nidogen polyclonal rabbit antibody for demonstration of entactinin a dilution of 1:200.

To detect a variety of antigens present in haematopoietic and lymphoid cells of the fetal spleens, a selected panel of antibodies was employed as follows: anti-myeloperoxidase polyclonal rabbit antibody for demonstration of cells of the granulocytic lineage in a dilution of 1:500 anti-glycophorin C monoclonal mouse antibody for detection of erythroid lineage cells in a dilution of 1:100 , anti-CD61 monoclonal mouse antibody for demonstration of megakaryocytesin a dilution of 1:100, CD20 monoclonal mouse antibody for detection of lymphoid cells of B cell lineage in a dilution of 1:400 (DAKO, Denmark A/S, M0755), CD3 monoclonal mouse antibody for detection of lymphoid cells of T cell lineage in a dilution of 1:100 and CD34 monoclonal mouse antibody for the detection of progenitor haematopoietic stem cells in a dilution of 1:50.

Serial semi-thin sections (four μm) were dewaxed with xylene, and bathed consecutively in descending ethanol series. Endogenous peroxidase activity was quenched by 15 minutes incubation of slides with 0.3% H2O2.). Slides were washed in phosphate buffer saline (PBS) for five minutes and then blocking reagent

Table 1. — *Extracellular matrix structural glycoproteins (tenascin-C, laminin, and entactin-nidogen) expression in the human fetal splenic parenchymal tissue (reticular cells-fibres, blood vessels). Correlation with fetal membrane changes. 10th to 12th week gestational age.*

Number of cases	Histological changes of fetal membranes	Tenascin-C expression				Laminin expression				Entactin (nidogen) expression			
		0+ (%)	1+ (%)	2+ (%)	3+ (%)	0+ (%)	1+ (%)	2+ (%)	3+ (%)	0+ (%)	1+ (%)	2+ (%)	3+ (%)
10	Normal histology of fetal membranes				10 (100)				10 (100)				10 (100)
10	Mild changes of acute chorioamnionitis				10 (100)				10 (100)				10 (100)
10	Moderate changes of acute chorioamnionitis				10 (100)				10 (100)				10 (100)
10	Severe changes of acute chorioamnionitis				10 (100)				10 (100)				10 (100)

was added for ten minutes. Slides were then incubated for 60 minutes in a humidified atmosphere with one of the monoclonal/polyclonal antibodies as follows: before incubation with primary antibodies for 90 minutes at 37°C. Slides were washed three times in PBS, before adding secondary reagents.Finally, bound antibody complexes were stained for ten minutes with 0.05% diaminobenzidine chromogen. Finally, sections were briefly counterstained with Mayer's haematoxylin and mounted. A homogenous, light brown staining revealed positive cells.The specificity and the pattern of each antibody were tested on positive control tissue samples according the manufacturers' technical data.

For the extracellular matrix structural glycoproteins (tenascin-C, entactin, and laminin), the sections were scored using a semiquantitative system based on the frequency of immunohistochemical reactivity of individual parenchymal elements as follows: 0, no detectable reactivity; 1+, mild number of cells and fibres reactivity; 2+ moderate number of cells and fibres reactivity; 3+ number of cells and fibres reactivity. For the immunohistochemical analysis of ECM molecules, the authors focused on the reticular cells and reticular fibres of the red and white pulp of the spleen. That is: the reticular cells and fibres marginating the red pulp, the reticular fibres of the splenic sinuses, reticular cells and fibres of the tissue surrounding the immature central arteries and neighbouring blood vessels of the white pulp, the splenic capsule, and splenic trabeculae. Special mention was given for tenascin-C immunoreactivity in marginal zone sinuses, red pulp vessels, and periarterial lymphatic sheaths and for laminin and entactin immunoreactivity in the basement membrane of vessels.

All immunostained sections were analyzed in a blind fashion without knowledge of the clinicopathological data and scored with an ×40 objective. The distribution of the ECM antibodies within the cells and the reticular fibres was recorded. Every stained cell and fibre was scored as positive regardless of staining intensity. To count the number of positive cells, a 10×10 square calibrated grid was inserted into the eyepiece of a BX40 binocular microscope. The sections were examined independently by two observers, and positive cellular and fibre staining for each antibody was manifested as fine yellow cytoplasmic granularity. For the immunohistochemical analysis of haematopoietic cells, the authors focused on the sinuses of the red pulp of the splenic parenchyma where haematopoiesis takes place, and for the lymphoid cells, they focused on the surrounding the central arteries, immature tissue, and smaller vessels of the white pulp, where the immune system is installed. For the quantitative assessment of the positive haematopoietic and lymphoid cells, the results were given per square millimetre in sections of fetal spleen.

It should be noted that the histologic diagnosis of chorioamnionitis was made on H&E-stained sections of the fetal membranes and carried out according to a standardized protocol with particular reference of the grade of inflammatory infiltrates by polymorphonuclear leukocytes (mild, moderate, and severe) at the level of the lesional tissue of the chorioamniotic plate.

Results

Immunohistochemical expression of ECM structural glycoproteins (tenascin-C, entactin, and laminin) in the reticular cells and fibres of the human fetal splenic parenchymal tissue was in correlation with fetal membranes changes.

10th to 12th week of gestation (first trimester of gestation)

During this period, the spleens in both settings (fetuses with chorioamnionitis and those after voluntary abortion), showed a strong TN-C immunoreactivity (3+), in all of the present cases. Reactivity was especially demonstrated in the reticular cells and fibres in marginal zone sinuses and red pulp vessels, splenic trabeculae, and capsule. A similar immunohistochemical pattern was demonstrated for laminin and entactin in the basement membrane of blood vessels of the spleen (Table 1).

13th to 24th week of gestation (second trimester of gestation)

During this period of development, a mild to moderate reduction of reticular cells and fibres for tenascin-C immunoreactivity was observed, in comparison with the findings of the control subjects. The reduction concerned mainly the reticular fibres of the marginal zone sinuses and red pulp vessels and lesser in the reticular cells and fibres marginating the splenic trabeculae and capsule. Similar changes showed the immunohistochemical analysis for laminin and entactin in the basement membrane of blood vessels of the spleen. The authors focused on the immunohistochemical analysis for tenascin-C in the reticular fibres of the splenic sinuses, and for laminin and entactin in the basement membrane of blood vessels. The present results in

Table 2. — *Extracellular matrix structural glycoproteins (tenascin-C, laminin, and entactin-nidogen) expression in the human fetal splenic parenchymal tissue (reticular cells-fibres, blood vessels). Correlation with fetal membrane changes. 13th to 24th week gestational age.*

Number of cases	Histological changes of fetal membranes	Tenascin-C expression				Laminin expression				Entactin (nidogen) expression			
		0+ (%)	1+ (%)	2+ (%)	3+ (%)	0+ (%)	1+ (%)	2+ (%)	3+ (%)	0+ (%)	1+ (%)	2+ (%)	3+ (%)
10	Normal histology of fetal membranes	-	-	-	10 (100)	-	-	-	10 (100)	-	-	-	10 (100)
10	Mild changes of acute chorioamnionitis	-	5 (50)	3 (30)	2 (20)	-	5 (50)	2 (20)	3 (30)	-	2 (20)	5 (50)	3 (30)
10	Moderate changes of acute chorioamnionitis	-	4 (40)	3 (30)	3 (30)	-	4 (40)	2 (20)	4 (40)	-	4 (40)	4 (40)	2 (20)
10	Severe changes of acute chorioamnionitis	-	3 (30)	4 (40)	3 (30)	-	2 (20)	2 (20)	6 (60)	-	3 (30)	3 (30)	4 (40)

Table 3. — *Extracellular matrix structural glycoproteins (tenascin-C, laminin, and entactin-nidogen) expression in the human fetal splenic parenchymal tissue (reticular cells-fibres, blood vessels). Correlation with fetal membrane changes. 25th to 32nd week gestational age.*

Number of cases	Histological changes of fetal membranes	Tenascin-C expression				Laminin expression				Entactin (nidogen) expression			
		0+ (%)	1+ (%)	2+ (%)	3+ (%)	0+(%)	1+(%)	2+(%)	3+(%)	0+(%)	1+(%)	2+(%)	3+(%)
10	Normal histology of fetal membranes	-	-	-	10 (100)	-	-	-	10 (100)	-	-	-	10 (100)
10	Mild changes of acute chorioamnionitis	-	3 (30)	4 (40)	3 (30)	-	4 (40)	4 (40)	2 (20)	-	4 (40)	5 (50)	1 (10)
10	Moderate changes of acute chorioamnionitis	-	3 (30)	2 (20)	5 (50)	-	6 (60)	2 (20)	2 (20)	-	4 (40)	5 (50)	1 (10)
10	Severe changes of acute chorioamnionitis	-	6 (60)	2 (20)	2 (20)	-	5 (50)	3 (30)	2 (20)	-	5 (50)	2 (20)	3 (30)

the ten cases featuring the fetal membranes mild changes of acute chorioamnionitis showed for tenascin-C, a mild in five, a moderate in three, and a strong expression in the remaining two; for laminin a mild in five, a moderate in two, and a strong expression in the remaining three, and for entactin a mild in two, a moderate in five, and a strong expression in the remaining three, respectively. The immunohistochemical analysis of the present ten cases showing in the fetal membranes moderate changes of acute chorioamnionitis revealed for tenascin-C, a mild in four, a moderate in three, and a strong expression in the remaining three; for laminin, a mild in four, a moderate in two, and a strong expression in the remaining four, and for entactin, a mild in four, a moderate in four, and a strong expression in the remaining two, respectively. Finally, the immunohistochemical analysis of our ten cases featuring to the fetal membranes severe changes of acute chorioamnionitis, showed for tenascin-C, a mild in three, a moderate in four, and a strong expression in the remaining three; for laminin a mild in two, a moderate in two, and a strong expression in the remaining six, respectively, and for entactin showed a mild in three, a moderate in three, and a strong expression in the remaining four, respectively (Table 2).

25th to 32nd week of gestation

During this period of development a further reduction of cells and fibres expressing tenascin-C, laminin, and entactin was observed, in comparison with the findings of the control subjects of the third trimester. Especially, the immunohistochemical analysis in the ten cases featuring the fetal membranes mild changes of acute chorioamnionitis showed for tenascin-C, a mild in three, a moderate in four, and a strong expression in the remaining three; for laminin, a mild in four, a moderate in four, and a strong expression in the remaining two, respectively, and for entactin showed a mild in four, a moderate in five, and a strong expression in the remaining one, respectively. The immunohistochemical analysis of the present cases featuring in the fetal membranes moderate changes of acute chorioamnionitis showed for tenascin-C, a mild in three, a moderate in two, and a strong expression in the remaining five; for laminin, a mild in six, a moderate in two, and a strong expression in the remaining two, respectively, and for entactin a mild in four, a moderate in five, and a strong expression in the remaining one, respectively. Finally, the immunohistochemical analysis of the present cases featuring the fetal membranes severe changes of acute chorioamnionitis showed for tenascin-C, a mild in six, a moderate in two, and a strong

Table 4. — *Reactivity of antibodies with heamatopoietic and lymphoid cells in the human fetal splenic parenchymal tissue. Correlation with fetal membrane changes (number of positive cells per square millimeter in sections of fetal spleen). 10th to 12th week gestational age.*

Number of cases	Histological changes of fetal membranes	Erythroid cells (glycophorin C)	Granulopoietic Cells (myeloperoxidase)	Haematopoietic cells (CD34)	Megakaryocytes (CD61)	B-lymphoid cells (CD20)	T-lymphoid cells (CD3)
10	Normal histology of fetal membranes	2750 ± 520 (range: 2150 to 4200)	35 ± 16 (range: 10 to70)	42 ± 17 (range: 16 to 83)	4 ± 2 (range: 2 to 5)	5 ± 2 (range: 3 to 8)	2 ± 1 (range: 1 to 4)
10	Mild changes of acute chorioamnionitis	2760 ± 510 (range: 2140 to 4300)	34 ± 15 (range: 11 to 75)	43 ± 12 (range: 16 to 80)	5 ± 1 (range: 2 to 7)	6 ± 2 (range: 2 to 9)	2 ± 1 (range: 1 to 4)
10	Moderate changes of acute chorioamnionitis	2810 ± 515 (range: 230 to 4100)	37 ± 13 (range: 11 to 80)	44 ± 13 (range: 18±80)	5 ± 1 (range: 2 to 7)	5 ± 2 (range: 2 to 8)	2 ± 1 (range: 1 to 4)
10	Severe changes of acute chorioamnionitis	2800 ± 510 (range: 2250 to 4100)	36 ± 12 (range: 12 to 75)	40 ± 15 (range: 16 to 80)	5 ± 1 (range: 2 to 8)	5 ± 2 (range: 2 to 8)	2 ± 1 (range: 1 to 4)

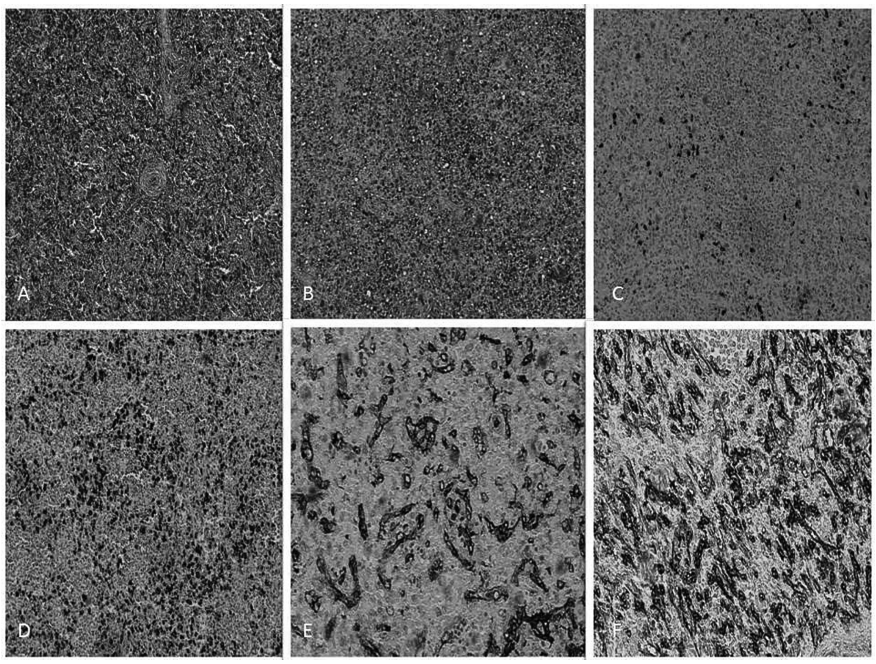

Figure 1. — Representative immunohistochemical staining micrographs for glyphorin-c (A, B), MPO (C, D), and CD34 (E, F) in non-infected and infected cases, respectively (original magnification ×100).

expression in the remaining two; for laminin a mild in five, a moderate in three, and a strong expression in the remaining two, and for entactin showed a mild in five, a moderate in two, and a strong expression in the remaining three, respectively (Table 3).

No important differences were found in the expression of ECM molecules in reticular cells and fibres in the splenic parenchyma marginating the splenic capsule and trabeculae.

10th to 12th week of gestation

Immunohistochemical analysis of the spleen samples during this period, showed in both cases (fetuses with chorioamnionitis and those after voluntary abortion), an approximately equal percentage of haematopoietic and lymphopoietic positive cells within the splenic sinuses (Table 4). The majority of these cells were arranged in aggregates and corresponded to the erythroid lineage showing a positive expression for glycophorin C (Figures 1A, B). Especially, erythropoietic cells averaged 2750 ± 520 cells/mm^2 (range: 2150 to 4200 cells/mm^2). Granulopoietic cells showing a positive expression for myeloperoxidase (Figures 1C, D), averaged 35 ± 16 cells/mm^2(range: 10 to 70 cells/mm^2). Progenitor haematopoietic stem cells showing a positive expression for CD34 by the endothelial cells of the splenic sinuses and stromal cells (Figures 1E, F), averaged 42 ± 17 cells/mm^2 (range 16 to 83 cells/mm^2). The immunohistochemical control CD61 to identify the megakaryocytes averaged 4 ± 2 cells/mm^2 (range 2 to 5 cells/mm^2). Finally, the B lymphopoietic lineage, CD20 positive cells (Figures 2A, B), showed an average of 5 ± 2 cells/mm^2 (range 3 to 8 cells/mm^2), whereas single cells expressing T lymphopoietic lineage, CD3 positive cells (Figures 2C, D), were detected in the perivascular loose connective

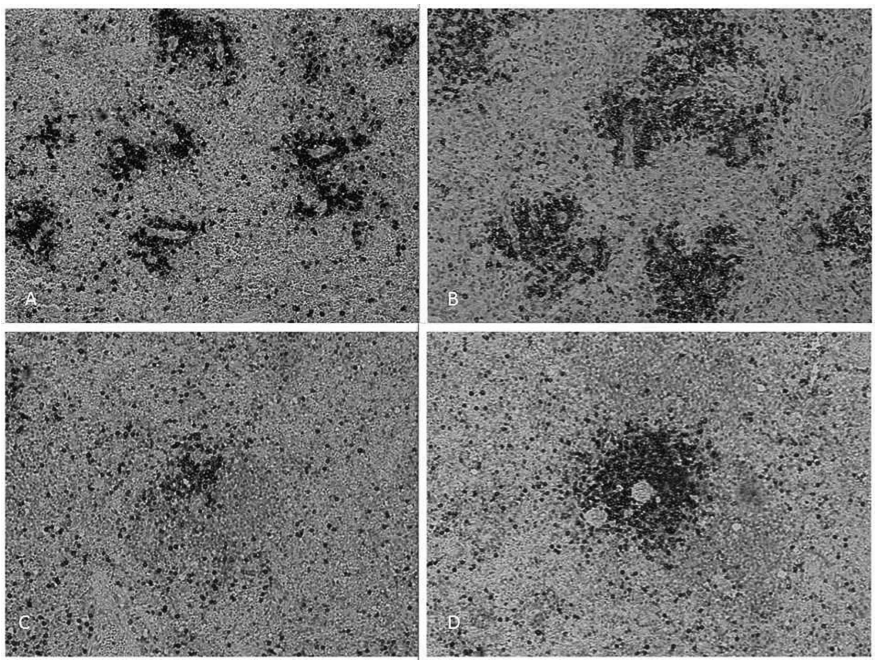

Figure 2. — Representative immunohistochemical staining micrographs for CD20 (A, B) and CD3 (C, D) in infected and non-infected cases, respectively original magnification ×100).

Table 5. — *Reactivity of antibodies with haematopoietic and lymphoid cells in the human fetal splenic parenchymal tissue. Correlation with fetal membranes changes (number of positive cells per square millimeter in sections of fetal spleen). 13th to 24th weeks gestational age.*

Number of cases	Histological changes of fetal membranes	Erythroid cells (glycophorin C)	Granulopoietic cells (Myelope-roxidase)	Haematopoietic cells (CD34)	Megakaryocytes (CD61)	B-lymphoid cells (CD20)	T-lymphoid cells (CD3)
10	Normal histology of ftal membranes	4100 ± 610 (range: 2900 to 5100)	42 ± 15 (range: 8 to 85)	52 ± 18 (range: 4 to 90)	8 ± 2 (range: 1 to 15)	48 ± 4 (range: 9 to 90)	18 ± 2 (range: 4 to 50)
10	Mild changes of acute chorioamnionitis	4120 ± 585 (range: 2850 to 5200)	215 ± 15 (range: 8 to 390)	55 ± 17 (range: 3 to 92)	9 ± 1 (range: 1 to 16)	49 ± 3 (range: 3 to 95)	19 ± 2 (range: 3 to 48)
10	Moderate changes of acute chorioamnionitis	4150 ± 586 (range: 2840 to 5100)	220 ± 15 (range: 9 to 385)	58 ± 15 (range: 9 to 90)	8 ± 2 (range: 1 to 15)	52 ± 4 (range: 8 to 98)	19 ± 2 (range: 3 to 46)
10	Severe changes of acute chorioamnionitis	4200 ± 580 (range: 2860 to 5125)	225 ± 15 (range: 9 to 380)	53 ± 15 (range: 9 to 85)	9 ± 2 (range: 1 to 15)	50 ± 2 (range: 3 to 90)	18 ± 2 (range: 4 to 50)

tissue, with an average of 2 ± 1 cells/mm^2 (range 1 to 4 cells/mm^2).

13th to 24th week of gestation

During this period within the splenic parenchyma in both settings, no significant quantitative changes were noted except in the granulopoietic and haematopoietic cells (Table 5). The splenic tissue in the cases of fetuses after voluntary abortion showed an average of granulopoietic cells 42 cells/mm^2 (range 8 to 85 cells/mm^2); in the cases featuring infection showed an average of 215 cells/mm^2 (range 8 to 390 cells/mm^2). The number of granulopoietic cells was more than five times higher than in the non-infected cases ($p < 0.05$). An increase of progenitor/stem haematopoietic CD34 cells was observed as well. No significant changes in the number of megakaryocytic CD61 cells, B or T lymphoid CD20 and CD3 cells respectively, were noted.

25th to 32nd week of gestation

In this period, the present series featuring chorioamnionitis showed a significantly higher and gradual increase in the number of granulopoietic (myoloperoxidase) positive cells in the mild cases of acute chorioamnionitis to the severe ones, in comparison with the control groups ($p < 0.05$). In addition, the number of progenitor haematopoietic stem (CD34 positive) cells showed a mild increase by 25% in cases of fetuses featuring severe changes of acute chorioamninitis. On the other hand, a gradual decrease at the level of erythropoiesis (glycophorin C) by 35%, and megakaryocytic (CD61), was observed. During the third trimester the immune system of the spleen revealed a decrease of both B and T lymphocytes by 25% and 30%, respectively, in comparison with the results of the control subjects (Table 6).

Table 6. — *Reactivity of antibodies with haematopoietic and lymphoid cells in the human fetal splenic parenchymal tissue. Correlation with fetal membrane changes (number of positive cells per square millimeter in sections of fetal spleen). 25th to 32nd weeks gestational age.*

Number of cases	Histological changes of fetal membranes	Erythroid cells	Granulopoietic cells	Haematopoietic cells	Megakaryocytes	B-lymphoid cells	T-lymphoid cells
10	Normal histology of fetal membranes	4200 ± 580 (range: 2950 to 5120)	80 ± 12 (range: 9 to 120)	82 ± 5 (range: 12 to 165)	20 ± 2 (range: 2 to 44)	84 ± 5 (range: 9 to 180)	70 ± 4 (range: 8 to 180)
10	Mild changes of acute chorioamnionitis	4010 ± 630 (range: 2800 to5110)	195 ± 15 (range: 9 to 210)	83 ± 5 (range: 13 to 170)	18 ± 2 (range: 2 to 40)	72 ± 5 (range: 9 to 140)	65 ± 4 (range: 8 to 180)
10	Moderate changes of acute chorioamnionitis	3200 ± 560 (range: 2800 to 4900)	250 ± 12 (range: 12 to 410)	84 ± 5 (range: 13 to 160)	16 ± 3 (range: 3 to 35)	68 ± 3 (range: 19 to 120)	60 ± 3 (range: 7 to 150)
10	Severe changes of acute chorioamnionitis	2820 ± 580 (range: 1900 to 4800)	440 ± 15 (range: 12 to 816)	110 ± 5 (range: 12 to 210)	15 ± 2 (range: 3 to 30)	60 ± 2 (range: 9 to 130)	58 ± 2 (range: 6 to 120)

Discussion

In this study the authors provided evidence from the cases of the control subjects that tenascin-C, laminin, and entactin are important components of the ECM of the spleen parenchyma in developing fetuses. The ECM molecules are expressed by the mesenchymal reticular cells and reticular fibres, early during fetal development of the spleen, and are continued to be expressed until the third trimester. The demonstration of expression of large amounts of these proteins at the end of the first trimester of development suggests that these peculiar ECM molecules not only constitute the base of the framework of the splenic parenchyma, but also contribute to the function and local regulation of haematopoiesis and development of the immune system of the spleen.

In the present cases of fetuses featuring chorioamnionitis, inflammatory cytokines and toxins generated and released during the course of intrauterine inflammation seem to be the offending factor for the reduction of the ECM structural glycoproteins (tenascin-C, laminin, and entactin) in the human fetal splenic parenchyma. As a consequence, the decrease of ECM molecules seems to be the main cause for the disorders of haematopoiesis in the spleen and in effective installation of the immune system of the spleen.

The present authors' review in the literature showed that tenascin-C constitutes a critical component of the bone marrow microenvironment that is required for hematopoietic regeneration [41]. Experimentally, this has been demonstrated from the fact that the expression of tenascin-C was found to be dramatically upregulated during haematopoietic recovery after myeloablation. Tenascin-C is able to exert important immunomodulatory activities on B and T lymphocytes, since it contains both a cell-binding site and an anti-adhesive site [42], regulating in this way the passage of lymphocytes to from the circulation in the lymphoid organs [43-45]. On this basis, it is likely that alterations of tenascin-C protein expression in splenic parenchyma, as observed in fetuses featuring chorioamnionitis in this study, could have a role in the mutation of adhesive properties of this protein.

Laminin and entactin constitute major components of the basement membranes of the vessels and are synthesized at high levels during tissue growth and development. It has been shown through experiments that ECM protein laminin has been demonstrated in the reticular fibres of T-cell dependent areas of human lymph nodes [46, 47]. The experiments suggest that laminin is important for lymphocytic traffic, since anti-laminin treatment of rats partially prevents lymphocytes from entering lymph nodes [48]. On this basis, it is likely that reduction of laminin protein in the developing fetal splenic parenchyma could cause further decrease of lymphoid cells circulation in the spleen, resulting in ineffective installation of the immune system in the organ. In addition, inflammatory toxins and cytokines could inactivate the local factors to stimulate ECM molecules production by the mesenchymal cells of the fetal splenic parenchyma or/and inactivate ECM structural glycoproteins for co-regulation or co-operation with other ECM components resulting in spleen depletion [49].

The comparative study of the quantitative percentage of ECM molecules at the end of the first trimester of development, remained stable in both sets (fetuses featuring chorioamnionitis and those voluntary abortion-control subjects). Probably, adaptive factors have not been activated at this early stage of development.

A recent in-depth review of the literature revealed that the majority of fetuses exposed to chorioamnionitis develop a systemic inflammatory response known as the fetal inflammatory response syndrome (FIRS) [50, 51]. Chorioamnionitis affects multiple organ systems such as the heart, causing abnormal fetal cardiac function [52], the brain causing developmental delay and lifelong neurological impairments [53 – 55], the lungs causing a reduced risk of developing respiratory distress syndrome (RDS) and an increased risk of bronchopulmonary dysplasia (BPD) [56], the retina causing retinopathy of prematurity (ROP) [57, 58], the kidneys causing oligohydramnios and resulting in reduction of fetal renal function, and finally it is obvious that uncontrolled situation could lead to sepsis [59– 60].

Recently, Galinsky *et al.*, have demonstrated that preterm fetal sheep exposed to intrauterine inflammation had a reduction in nephron number of approximately 20% [61]. Apparently, this is the first report in the literature in which complications of chorioamnionitis are associated with organ tissue reduction.

The continued release of ECM structural glycoproteins tenascin, laminin, and entactin molecules suggests that ECM synthesis and production are important for physiological events in the human fetal spleen, such as haematopoiesis during the second trimester of development, and installation of the immune system during the third trimester of development, respectively.

The close association of chorioamnionitis on the one hand, and reduction of the extracellular matrix structural glycoproteins (tenascin, laminin, and entactin) of the developing splenic parenchyma in association with disorders of haematopoiesis and immune system of the spleen, on the other hand, suggests a causal relationship, but the exact mechanism is uncertain. Further studies are required to determine the offending factors underlying the chorioamnionitis-induced reduction in ECM structural glycoproteins and eventual interplay of haemato-lymphopoietic disorders in the developing fetuses.

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