IMMUNOHISTOCHEMICAL DETECTION OF FIBRONECTIN IN HUMAN THYMUS-COMPARISON BETWEEN GROUPS OF DIFFERENT AGES

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Objective: To compare distribution of fibronectin content in various parts of human thymus between groups of different ages using immunohistochemistry. **Design:** Comparative study. **Place and duration of study:** The study was carried out in Anatomy Department, Army Medical College Rawalpindi from Oct 2007 to Dec 2007. **Material and methods:** Forty specimens from tissue sections of human thymus were separated into two groups with 20 specimens in each group: Group A consisted of specimens from the patients of 1-25 years and Group B of specimens from patients beyond 40 years. These specimens were fixed in 10% formalin solution and processed for paraffin embedding. Five micron thick sections were made. Immunohistochemical staining was utilized to localize fibronectin in various regions of human thymus (capsule, connective tissue between lobules, cortex, medulla and areas around blood vessels). **Results:** Statistically highly significant difference was found between two groups with a marked increase in the distribution of fibronectin content of the thymic capsule, the connective tissue between the lobules, areas around the blood vessels, and the medulla and cortex of the thymus in Group B compared to Group A. **Conclusion:** The fibronectin content in human thymus in its various regions shows a marked increase in aged people as compared to younger ones.

Keywords: Human thymus, Fibronectin, Aging, Immunohistochemistry

INTRODUCTION

Thymus is a lymphoid organ located in the mediastinum. It is almost fully developed at birth.[1,2] The age related change in thymus is a consistent phenomenon and has been proposed to be a primary event leading to the immunological aging.[3–5] It appears that the immune system is markedly involved with aging in animals.[6] The progenitor cells derived from bone marrow undergo different stages of maturation within the thymic microenvironment to produce functional T-cell repertoire.[5] This thymic microenvironment consists of epithelial cells and thymic extra-cellular matrix (ECM), which includes fibronectin, laminin, and collagen.[4]

The fibronectin usually exists as a dimer composed of two almost identical monomers covalently linked by a pair of disulphide bonds.[7] The fibronectin protein is the product of single gene. It can exist in several isoforms arising from alternative splicing of its pre-mRNA.[8] It has molecular weight of 450,000 Da. There are two types of fibronectin found on the basis of its solubility: a soluble plasma fibronectin and a less insoluble cellular fibronectin. The plasma fibronectin is a major protein component of blood plasma (300 µg/ml) and other body fluids. It is synthesized mainly in liver by the hepatocytes. The cellular fibronectin is a major component of insoluble extra-cellular matrix (ECM). It is predominantly secreted by fibroblasts as a soluble dimer and is assembled into the ECM, an insoluble network that separates and supports the organs and tissues of an organism.[9] It is widely found in connective tissue particularly at the interface between the basement membrane and the epithelial layer and, wall of blood vessels.[10] It is abundant in ECM of most of the soft tissues, skeletal muscles, only in traces in tendons and not found in mature cartilages.[11]

In vertebrate organisms, fibronectin has numerous functions to perform. It mediates a large number of cellular interactions with ECM and has important role in cell adhesion, growth, migration and differentiation.[4,9,10,12,13] A critical role of fibronectin in wound healing has been identified when plasma fibronectin, along with fibrin is deposited at the site of injury forming a blood clot and hence stoppage of bleeding.[14] Fibronectin has also been found to be necessary for embryogenesis.[13]

Although fibronectin has been under study for more than two decades, this remarkable complex molecule is still the subject of exciting deliveries. The thymic involution with advancing age involves changes in its histological structure. Thymus has also been investigated for age related change in its fibronectin content. In an animal study, an increase in the fibronectin content of various parts of thymus was found in the rats with advancing age.[4] The biosynthesis of fibronectin has been reported to increase from 2 months to 22 months in mouse skin[15] and on cultured pig fibroblasts.[16]

The review of published medical literature

reveals that most of the studies on changes in thymus with aging have been performed in mice and other animals and the literature about human thymus especially relevant to this part of the world is limited. Moreover despite considerable importance of ECM and its fibronectin content, the data available on this aspect is scarce.

This study has been planned to investigate changes in the distribution of fibronectin content of human thymus by immunohistochemical staining in two groups of different ages.

MATERIAL AND METHODS

Tris Phosphate buffer solution (TBS), Citrate buffer, Hydrogen peroxide (H_2O_2), Di-aminobenzidine tetrahydrochloride (DAB), primary antibody-specific for fibronectin (mouse anti-human antibody), normal horse serum (NHS), biotinlayted secondary antibody and Streptavidine peroxidase conjugate) were procured from local distributor of Novocastra.

Forty specimens from tissue sections of human thymus were obtained from Armed Forces Institute of Cardiology Rawalpindi during open chest surgery. The specimens obtained were separated into two groups with 20 specimens in each group. In Group A the mean age was 9.57±7.75 years with a male female ratio of 10:10. Out of these 5 were Tetrology of Fallot (25%), 1 was Atrial septal defect (5%), 2 were Mitral stenosis (10%), 2 were Mitral regurgitation (10%), 1 was Mitral valve insufficiency (5%), 1 was Ventricular septal defect (5%), 1 was Coarctation of aorta (5%), 1 was Vegetation (5%), Glenn's shunt (5%), Tricuspid Atresia (5%). In Group B the mean age was 55.7±7.63 years with a male female ratio of 19:1. All these cases were of coronary artery disease. It was considered unnecessary to include specimens from patients of age between 26–39 years because the study was not concerned with correlation of study variables with age progression rather it aimed to compare changes in these variables at different ages. Tissue sections of specimens were placed for 48 hours in numbered bottles containing 10% formalin solution. These formalin fixed samples were embedded in paraffin wax. 5-micron thick sections were made on rotary microtome. The sections were then deparaffinized and stained by labelled streptavidine biotin (LSAB) method.[17] In this method, there are three layers of antibodies. Unlabelled primary antibody specific for fibronectin is the first layer, biotinylated secondary antibody, the second layer and streptavidine peroxidase conjugate, the third layer. The primary antibody was prepared by adding distilled water to the contents of the company provided bottle to make 1 ml of fluid. 10 µl of prepared antibody were added to 990 µl of diluent to make 1:100 dilutions to be used in procedure. The sections were deparaffinized and hydrated, then washed in TBS. The sections were kept in 3% v/v H_2O_2 in tap water for 10 minutes and then washed again, first in distilled water and then in TBS. The slides were heated in citrate buffer in microwave oven for one min. After bringing it to the room temperature, slides were washed in TBS again. The sections were incubated at 25 °C first in blocking serum (NHS) for 25 min, then in primary antibody for one hour, then in secondary antibody for 20 min followed by in stretavidine peroxidase conjugate. Between these steps the sections were washed in TBS every time. The sections were then incubated at 25 °C in DAB (as chromogen) for 20 min. After rinsing in TBS and tap water the sections were counterstained with haematoxylin, dehydrated, cleared and mounted. The sections were then examined and evaluated for fibronectin content in capsule, connective tissue between the lobules, areas around blood vessels and in the medulla and cortex of thymus in three randomly selected fields of each section under 40× objective. Intensity of immunohistochemical staining of fibronectin was defined as: negative= (-), Faint= (±), weakly positive= (+), positive= (++), clearly positive= (+++), strongly positive= (++++) (Table-1).[4] The sections of thymus from 2, 15, 25 and 50 years old patients were chosen and findings about these were tabulated.

Stastical Analysis

The data was analyzed using computer software SPSS 11.0. The statistical difference of ordinal variables between the groups was assessed by Chi-Square test. The difference was regarded as statistically significant if the *p*-value was less than 0.05.

RESULTS

The localization of fibronectin in thymic capsule in Group A specimens was; weakly positive (+) in 8, positive (++) in 9, clearly positive (+++) in 3 cases. There was no section with strongly positive staining (++++) in Group A. In contrast to it the fibronectin in Group B was; weakly positive (+) in none of the case, positive (++) in 2, clearly positive (+++) in 7 and strongly positive (++++) in 11 cases. Hence 55% of sections showed strongly positive (++++) for fibronectin in Group B wile no section in Group A exhibited such degree of staining. The difference between localization of fibronectin in the capsule of Group A and of Group B specimens was statistically highly significant ($p<0.001$, Table-1).

The localization of fibronectin in interlobular connective tissue in Group A specimens

was; weakly positive (+) in 8, positive (++) in 11, clearly positive (+++) in 1 case. There was no section with strongly positive staining (++++) in Group A. In contrast to it the fibronectin in Group B was; weakly positive (+) in none, positive (++) in 4, clearly positive (+++) in 7 and strongly positive (++++) in 9 cases. Forty-five percent of sections showed strongly positive (++++) for fibronectin in Group B wile no section in Group A showed such degree of staining. The difference between localization of fibronectin in interlobular connective tissue of Group A and of Group B specimens was statistically highly significant (p<0.001, Table-1).

The localization of fibronectin in medulla in Group A specimens was; negative (–) in 1, faint (±) in 8, weakly positive (+) in 9, positive staining (++) in 2 cases. Clearly positive (+++) and strongly positive staining (++++) was not present in any case. In contrast to it the evaluation of fibronectin in Group B was; weakly positive (+) in 3, positive (++) in 11, clearly (+++) in 6 and none with strongly positive (++++) staining. Although the staining in Group B sections was more intense as compared to those in Group A but none of the specimens exhibited strongly positive (++++) staining. The difference between localization of fibronectin in medulla of the Group A and of Group B specimens was statistically highly significant (p<0.001, Table-1).

The localization of fibronectin in cortex in Group A specimens was; negative (–) in 11, faint positive (±) in 3, weakly positive (+) in 6 cases. There was no section with positive (++), clearly positive (+++) and strongly positive staining (++++) in Group A. In contrast to it the evaluation of fibronectin in Group B was; weakly positive (+) in 11, positive (++) in 9 cases. There was no section which showed clearly positive (+++) and strongly positive (++++) staining. The difference between localization of fibronectin in cortex of Group A and of Group B specimens was statistically highly significant (p<0.001, Table-1).

The localization of fibronectin in the areas around blood vessels in Group A specimens was: weakly positive (+) in 7, positive (++) in 9, clearly positive (+++) in 4 cases. There was no section with strongly positive staining (++++) in Group A. In contrast to it the evaluation of fibronectin in Group B was: weakly positive (+) in none, positive (++) in 3, clearly positive (+++) in 6 and strongly positive (++++) in 11 cases. Hence 55% of sections showed strongly positive (++++) for fibronectin in Group B wile no section in Group A exhibited such degree of staining. The difference between localization of fibronectin in areas around blood vessels of the Group A and of Group B specimens was statistically highly significant (p<0.001, Table-1).

The sections from specimens of 2, 15, 25 and 50 years of patients chosen already were specifically evaluated for distribution of fibronectin in various regions of thymus (Table-2, Figures: 1–5).

The comparison within Group A revealed differences in distribution of fibronectin between sections from male and female patients but that was statistically insignificant (p>0.05, Table-3). Such a comparison was not possible in Group B as sufficient number of specimens from female patients could not be made available.

Table-1: Comparison of immunolocalization of fibronectin in different regions of thymus between groups

Intensity of labelling	Group A					Group B				
	Thymic capsule	Interlobular connective tissue	Medulla	Cortex	Areas around blood vessels	Thymic capsule	Interlobular connective tissue	Medulla	Cortex	Areas around blood vessels
(-)	0	0	1	11	0	0	0	0	0	0
(±)	0	0	8	3	0	0	0	0	0	0
(+)	8	8	9	6	7	0	0	3	11	0
(++)	9	11	2	0	9	2	4	11	9	3
(+++)	3	1	0	0	4	7	7	6	0	6
(++++)	0	0	0	0	0	11	9	0	0	11
Total	20	20	20	20	20	20	20	20	20	20

Statistical significance of difference between male and female:
thymic capsule: p<0.001; interlobular connective tissue: p<0.001; medulla: p<0.001; cortex: p<0.001; areas around blood vessels: p<0.001

Table-2: Immunolocalization of fibronectin in human thymus of different ages

Age	Thymic capsule	Interlobular Connective tissue	Areas around blood vessels	Cortex	Medulla
2 years	+	+	+	-	±
15 years	++	++	++	±	+
25 years	+++	+++	+++	+	++
50 years	++++	++++	++++	++	++

Intensity of labelling defined as:
Negative= (-), Faint= (±), Weakly positive= (+), Positive= (++), Clearly positive= (+++), Strongly positive= (++++)

Table-3: Comparison of immunolocalization of fibronectin in different regions of thymus between group A male and female cases

Intensity of labelling	Male					Female				
	Thymic capsule	Interlobular connective tissue	Medulla	Cortex	Areas around blood vessels	Thymic capsule	Interlobular connective tissue	Medulla	Cortex	Areas around blood vessels
(-)	0	0	0	4	0	0	0	1	7	0
(±)	0	0	4	2	0	0	0	4	2	0
(+)	5	5	6	4	5	6	6	4	1	3
(++)	4	5	0	0	4	3	3	1	0	5
(+++)	1	0	0	0	1	1	1	0	0	2
(++++)	0	0	0	0	0	0	0	0	0	0
Total	10	10	10	10	10	10	10	10	10	10

Male and female statistical significance of difference between:
thymic capsule: $p>0.05$; interlobular connective tissue: $p>0.05$; medulla: $p>0.05$; cortex: $p>0.05$; areas around blood vessels: $p>0.05$

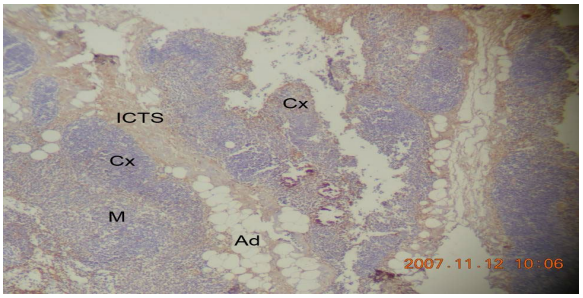

Figure-1: 2 Years

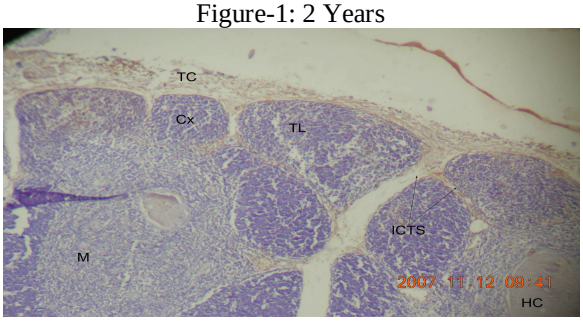

Figure-2: 15 Years

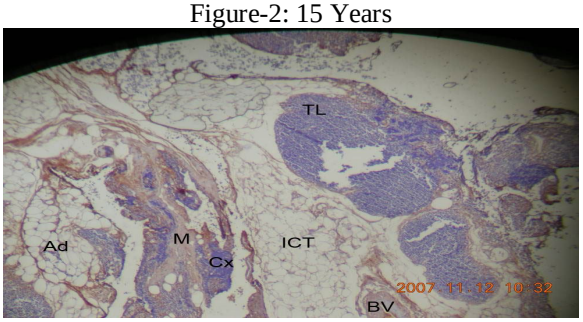

Figure-3: 25 Years

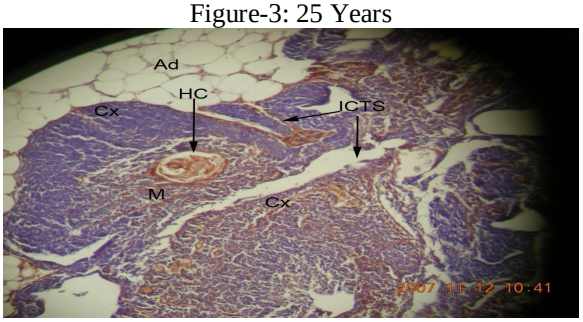

Figure-4: 50 Years

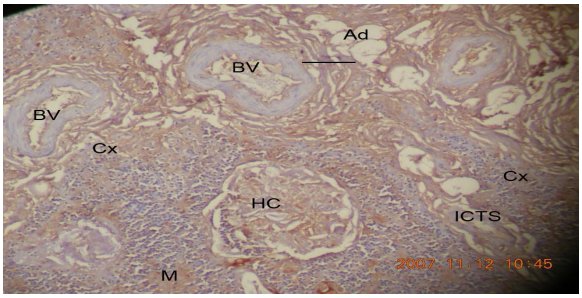

Figure-5: 50 Years

Microphotographs showing the distribution of fibronectin in thymus at different ages

DISCUSSION

In this immunohistochemical study, statistically highly significant increase in the distribution of fibronectin content was found in various regions of human thymus with advancing age. It is consistent with an earlier study[4] reporting a similar increase in fibronectin content of rat thymus. The pattern of this age related increase in intensity of staining varies in different regions of human thymus. In sections from Group B strongly positive staining (++++) was found in thymic capsule (in 55% 0f sections), interlobular connective tissue (in 45% of sections) and in areas around blood vessels (in 55% of sections); while in Group A no section showed strongly positive staining (++++). As for as the regions of medulla and cortex are concerned: in Group B, clearly positive staining (+++) was seen in the medulla (in 30% of sections) and positive staining (++) was seen in cortex (in 45% of cases) while in Group A no section showed clearly positive staining (+++) in medulla and positive staining (++) in cortex. The increase found in the immunohistochemical detection of fibronectin in different regions of human thymus with advancing age is in line with results of the studies discussed here.

The level of fibronectin and type III collagen mRNA was found to be raised during in vitro ageing of pig skin fibroblast.[16] The biosynthesis

of the fibronectin determined by immunoprecipitation of 35 S methionine labeled peptides in SDS extracts of mouse skin showed a progressive increase with advancing age.[18] A denser ECM pattern was seen in aged normal mice as compared to young adult normal animals.[15] The increased synthesis of fibronectin in aged fibroblasts was found explaining the mechanisms involved in aging and development. The increase appeared to have correlation with the general increase noticed in the rate of synthesis of protein per cell.[19] In an enzyme linked immunosorbent assay, the amount of cell surface fibronectin and the amount released into the culture medium in human skin fibroblasts during successive cultivation were investigated. Released fibronectin per cell showed a marked increase in late passage.[20] With advancing age an increase in the fibronectin content of the rat thymus was found along with changes in its histological structure.[4]

The increase found in the fibronectin content of human thymus with advancing age can be explained by important observations present in the literature; increase in the amount of connective tissue in capsule as well as interlobular area of thymus[15]; general increase in rate of protein synthesis in cultured human fibroblasts[19]; markedly rising levels of fibronectin and type III collagen mRNA[16].

Aging is a complex interaction of genetics, chemistry, physiology and behaviour. In aging immune system, T-lymphocytes take longer to replenish and their ability to function, declines. The functional differences were revealed when the fibronectin synthesized by human fibroblasts was analyzed at different times during serial sub-cultivation. The fibronectin that was isolated from late passage cells was found defective in promoting cell adhesion, cell spreading and focal contact formation.[21,22] This defect in the capability of fibronectin molecules secreted from aged cells may have role in aging process. The age related increase in the fibronectin content of human thymus has relevance with immunological aging and needs to be explored further.

As far as the gender based difference in immunohistochemical detection of fibronectin is concerned, sections in Group A were studied for it and a statistically insignificant difference was found between male and female patients. It has support from an earlier study where a significant increase in fibronectin concentration from 3rd to 4th decade of life was found with no difference between men and women.[23] This gender based study could not be extended to Group B as sufficient specimens from female patients in this group could not be arranged at that time.

CONCLUSION

The objective of comparing distribution of fibronectin in various regions of human thymus between two groups of different ages by immunohistochemistry has been met. The study shows that there is significant difference in human beings also as have been earlier reported in animals. There was marked increase in immunolocalization of fibronectin in human thymus with advancing age. A study with more available resources is suggested to deliberate upon incremental changes occurring with progressive age in both sexes in future.

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