Original Article

An Immunohistochemical Study of Retinal Collagen IV Expression during Pre- and Postnatal Periods in Balb/c Mice

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Abstract

Objective: Basement membranes are specialized extracellular matrices which play important roles such as cell regulation, proliferation and migration. Collagen fibers, especially type IV, are the most important basement membrane constituents. As retina is one of the target organs in diabetes mellitus, and nephropathy is a major cause of end stage renal and retinal diseases resulting in increased morbidity and mortality, early diagnosis leads to better treatment. Hence, in this investigation, the appearance and distribution of collagen IV during gestational days and early postnatal periods were observed.

Materials and Methods: 24 intact female Balb/c mice were kept under normal conditions. After mating, appearance of a vaginal plug was assumed as day zero of the pregnancy. From days 13-18 of gestation, the pregnant mice were euthanised and their embryos as well as pups from days 1 to 5 were collected. For histochemichal studies, heads of the specimens were fixed, serially sectioned and immunohistochemicall studies were performed by using monoclonal antibodies for tracing of collagen type IV.

Results: Our findings revealed that the amount of collagen IV in the internal limiting membrane (ILM) and extra cellular matrix (ECM) of the retina, as well as vessels of the vitreus body appear on embryonic day 16. Also, a patchy distribution was observed in the pigmented epithelium which continued to further develop until the end stage of embryonic life. Strong labeling was observed until postnatal day 3 but did not increase significantly thereafter.

Conclusion: These findings establish the importance of collagen IV during the critical period of retinal development. In addition, this study indicates that high levels of collagen IV are present in the basal membrane (BM) of the inner limiting membranes and pigmented epithelium (3rd post natal on the 3rd postnatal day.

Keywords: Collagen IV, Extracellular Matrix, Development, Retina, Mouse

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Introduction

The basement membrane (BM) is a specialized region of the extra cellular matrix that consists of different components such as collagen, laminin, fibronectin, sulfated and nonsulfated glycosaminoglycan (1). Among BM components, fibronectin, heparin sulfate and collagen type IV play critical roles in cell signaling and differentiation of the optic cup (2-4). In other words, major components of conducting signals in retinal development are also components of the extra cellular matrix (ECM) which have been organized into the basement membrane (5, 6). Some of these molecules bind to other cells, whereas others bind to the basement membrane of the developing retina where they are called the limiting membranes (7). Hence, it could be speculated that certain molecules and components of extra cellular matrix are necessary for cell differentiation (8, 9). Among these macromolecules, collagen type IV plays a major regulatory role in cell behaviors and developmental phenomena such as proliferation, migra-

tion, differentiation, morphogenesis and metabolism (10-12). The most prominent roles of ECM are migration and adhesion of cells to cells and their substrates (13). Collagen type IV serves these roles in both inner and outer limiting membranes of the retina and optic cup (14). Also, components of ECM such as the α_1-α_2 chains of collagen type IV are required for controlling blood circulation in the developing visual system (15) as this molecule is known to have an important role in eye physiology. For example, transparent areas of the eye are dependent on the correct development of ECM and special arrangement of collagen (16). Collagen type IV plays an important role in the morphogenesis and cell association during retinal development. It seems that appearance and synthesis of collagen can represent the development and differentiation of retina (17). In other words, morphogenesis of embryonic tissues such as the retina is made possible by intracellular mechanisms modulated by exogenous signals (18). In fact, growth factors and extra cellular matrix components act as mediators in cell development and differentiation (19). Because collagen type IV is an important component of extracellular and basement membranes (20), the aim of the presented study was to determine its appearance and distribution by labeling and tracing it during the critical period of optic cup and retinal development until the last day of pregnancy as well as after birth to more precisely investigate its role in retinal differentiation.

Materials and Methods

Animal procedure

In this study, 24 female Balb/c mice were obtained from animal house of Mashhad University of Medical Sciences. The environmental conditions were 23-25°C, relative humidity of 50-55% and 12 hour light-dark cycles. Mature females were mated overnight with males of the same strain (two females and one male) and examined the next morning for vaginal plugging. Females with vaginal plugs were designated day zero of pregnancy. Pregnant mice from gestational days 13-19 (E13 to E19) and pups of 1 to 5 postnatal days of age were euthanized and transcardinally perfused with physiological saline followed by formalin (Merck Pharmaceuticals, Germany); their brains were removed and postfixed for at least 24 hours at room temperature in the same fixative. The head of embryos and their removed eyeballs were routinely processed and embedded in paraffin with eight-micron sections being carried out serially. Only slides containing eyeball samples were used in this study. The sections were stained with cresyl violet or incubated with the anti-collagen IV monoclonal antibody (Sigma-Aldrich, USA). Photographs were taken using an Olympus BH2 light microscope.

Immunohistochemistry study

The avidin-biotin-peroxidase complex (ABC) was used for our immunohistochemical study. Sections were deparaffinized, rehydrated and washed twice for 5 minutes in a 0.05 M Tris buffer containing 1.5% NaCl, pH: 7.4. To block nonspecific antibodies, sections were preincubated in 0.3% Triton X-100 in TB-NaCl followed by 5% goat serum (Gibco, UK) for 2-3 hours; they were then left for 12-24 hours at 4°C to react with primary antibody anti-collagen (Sigma-Aldrich, USA) diluted 1:250 in TB-NaCl with 0.3% Triton X-100 and 2% bovine serum. The tissues were then washed three times with TB-NaCl for 10 minutes each time and incubated for 2 hours in biotinylated goat anti-rabbit IgG (1:400 in TB-NaCl). After three further rinses, each lasting 1 hour, the endogenus peroxidase activity was blocked by incubating the sections in 0.03% H_2O_2 in methanol for 30 minutes. The tissues were then incubated for 2 hours in 1:100 avidin-biotinylated horseradish peroxidase complex, each washed in TB-NaCl for 30 minutes three times, and finally reacted with a 0.03% solution of 3, 3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, USA) containing 0.03% H_2O_2 for 10-15 minutes before they were washed and lightly counterstained with hematoxylin and eosin (Sigma-Aldrich, USA). Subsequently, the tissues were washed again, air-dried, dehydrated and then mounted in PBS/glycerol (1:9). Using an Olympus BX51 light microscope, images were taken and the lens collagen IV reactions in different embryonic stages were evaluated.

Statistical analysis

The staining intensity was graded by two different individuals in accordance with Firth's method. The scoring gradation had four stages and reflected the severity of reaction from negative to weak, moderate, strong and very strong. P-values<0.05 were considered as significant.

Results

The immunohistochemical data collected until the early 15th day of gestation indicated no detectable collagen IV reaction at any area of the eyeball (Fig 1A). The first noticeable change was the appearance of a very pale reaction on the late 15th day of gestation which was distributed in the extra cellular matrix proteins of the retinal ganglion cells and

vitreous body (Fig 1B). On the 16th day of gestation, the retinal inner and external limiting membranes as well as hyaloid vessels of the vitreous body showed weak reactions (Fig 1C, D). Also, a patchy distribution was observed in the retinal pigment epithelium. This reaction continued and slightly increased in the following days (Table1, Fig 2). Strong labeling was observed in the first 3 postnatal days, but after this period, collagen IV reaction did not show distinct changes, further intensity of reaction was observed in the inner limiting membrane and pigmented epithelium.

Table 1: Distribution of collagen IV during retinal development in mouse ED (embryonic day), PND (postnatal day), ILM (inner limiting membrane), ECM (extra cellular matrix) and ELM (external limiting membrane). Collagen IV reaction intensity: no reaction (-), weak (+), moderate (++), high (+++) and severe (++++).

DE	ILM	ECM	ELM	BM/pigment epithelial cells
14	(-)	(-)	(-)	(-)
15	(+)	(-)	(+)	(++)
17	(++)	(+)	(++)	(+++)
3rd PN	(++++)	(+++)	(++)	(++++)

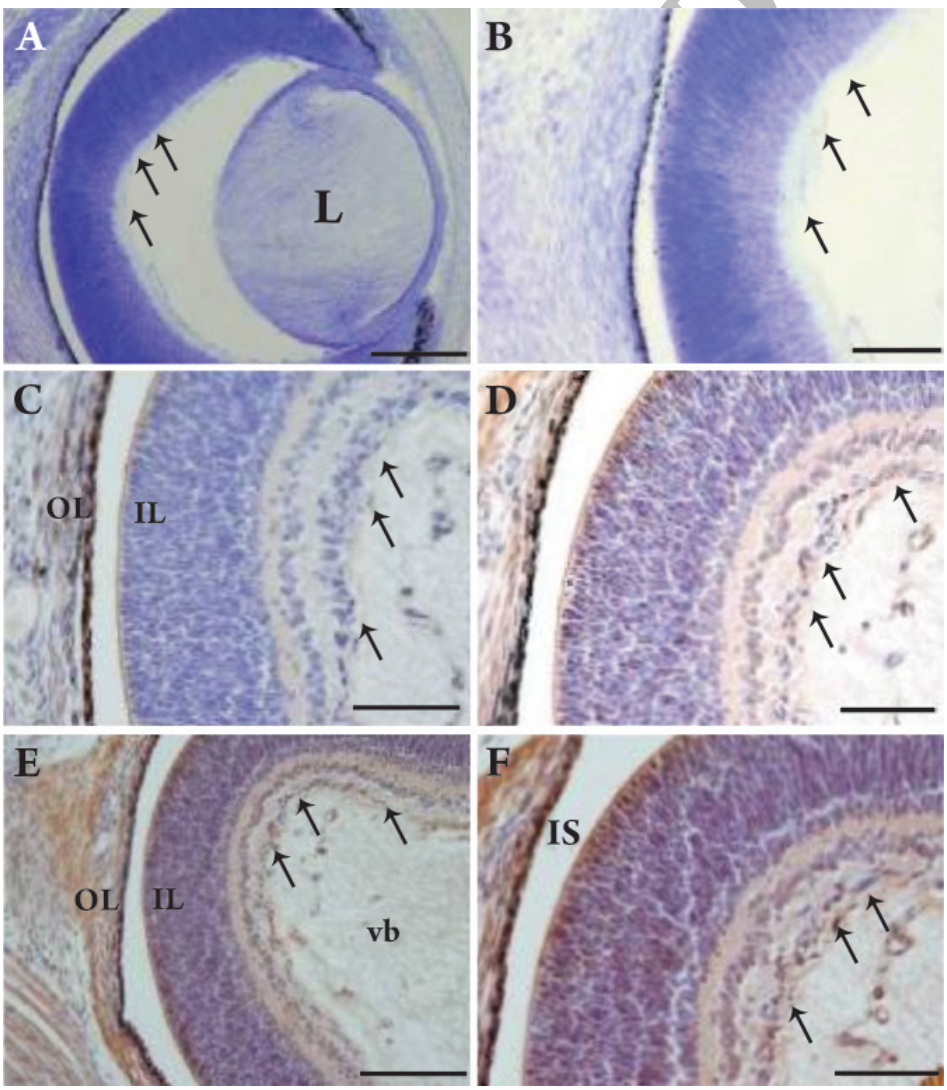

Fig 1: Retinal cross sections (15th gestation day) incubated with the collagen IV antibody (A) and with higher magnification (B), extra cellular matrix (ECM) and inner limiting membrane (ILM) indicate no reaction (arrows). On day 16 and 18 of gestation (C and D respectively) arrow-marked areas showed weak reaction. Also, on the third postnatal day, a high density of collagen IV fibrils was detected in the ILM along the vitreoretinal border as well as the pigmented layer (arrow). The vitreous body (VB) stroma also showed weak reaction. (E and F). Scale bar 100 μm.

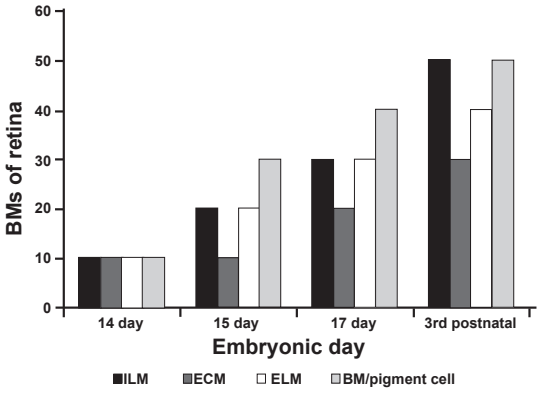

Fig 2: Immunohistochemical analysis of collagen IV during retinal development in mouse. Data used in quantification 10→ (-), 20→ (+), 30→ (++),40→ (+++) and 50 → (++++).

Discussion

The presented study shows that although the inner limiting membrane was formed by embryonic day 14, collagen type IV could not be found in any parts of the developing retina. Previous studies have shown that at this time, some of the epithelial tissues express collagen IV and that basement membrane begins to appear at its former area. The question was: why were we not able to detect this protein? It could be assumed that although collagen IV should appear at this time, it is possible that its concentration in contrast to other proteins in the basement membrane during the days to follow is undetectably low. The other possibility is the kind of antibody used in this study. In this investigation we preferred to use a monoclonal antibody as it binds with only one specific epitope of collagen IV (in contrast to a polyclonal antibody which binds with several epitopes of a protein); therefore, it is possible that based on this very specific reaction, the number of active sites were not satisfactorily detectable. It could be possible that if we used a polyclonal antibody, the rate of reaction could be higher. The first immunostaining was weakly detected on the late 15th embryonic day. This labeling was detected in different eye parts such as the inner and outer limiting membranes, and the optic cup on day 17. The development of collagen type IV at this stage represents the special role of cell to cell interaction in retinal layers. Differentiation of these layers into cell types reflects an important role in retinal development (21). Observing the labeling reactions indicated that cell interactions continued in the following days. The cell interaction intensity gradually increased in a way that they concluded at their highest possible degree on the 3rd day after birth. No remarkable change was recorded afterwards. Results of a similar study showed that the retinal development period is from 15.5 days of gestation to the 4th postnatal day (22). Also, developmental stages of rodents such as rats indicate that the final development of their visual organ and the differentiation of these layer of immature retinal cells from specialized ones occur in the last embryonic week particularly in the early stages of postnatal development (23). Investigations show that the retinal structure is completely formed from invagination of the two optic cup cell strata (24). The internal layer of the retine is covered with an inner limiting membrane which is adjacent to the vitreous body, whereas the outer limiting membrane is adjacent to the internal surface of the outer layer of the optic cup retina (25). The basement membrane formation is dependent on the existence of specific proteins among which collagen IV is the most predominant (26). Therefore, due to the fact that elimination of collagen type IV could result in deformation of the basement membrane, one can find the vital role of this type of protein in basement membrane formation (27). Besides, the basement membrane covering the internal layer of retinal cells (ganglionic cells) makes the adjacency of this layer to the developing vitreous body possible. The vitreous body, exchanging ions and molecules as well as developmental signals, helps in the deferentiation of the bacement membrane (28). This is of prime importance because some of the mutants and transgenic that can not expressed collagen have visual defect (29). The labeling of collagen in ECM of retinal cells, and particularly its high concentration in ganglionic cells showed that the ganglionic layer development is dependent on the inner limiting membrane and needs the collagen type IV producing ECM (30). Moreover, the outer limiting membrane, connecting the inner layer of the optic cup with the outer layer of the pigmented epithelium, guarantees extended cell interactions which help the formation and deferentiation of rod and cone cells as well as the retinal pigment epithelium (31).

Conclusion

Therefore, any disorder in the development of collagen IV in the optic cup affects the BM in this area and may result in anomalies such as defects in the natural development of pigmented epithelium or lack of accurate deferentiation

of rod and cone cells. Hence, it seems that retinal development depends on various molecular changes and different sugars and proteins such as laminin and fibronectin, as well as different kinds of collagen especially collagen type IV. In other words, the development of retina requires various outer cell compounds including the inner limiting membrane and the extra cellular matrix. These compounds are mainly dependent on their composing elements especially collagen type IV to develop and play an important part in deferentiation of cell layer.

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