Development of Bruch’s membrane in the chick: an electron microscopic study

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The development of fibrous connective tissue in Bruch’s membrane within the choroid of chronologically staged chick embryos was observed by means of transmission electron microscopy. The appearance of these connective tissue elements follows an orderly developmental sequence. The first component of Bruch’s membrane to appear is a continuous basal lamina at the basal surface of the presumptive retinal pigment epithelium (RPE) at 2½ days. Microfibrils and associated amorphous material are present in the adjacent connective tissue space. A discontinuous inner collagenous layer is observed at the basal aspect of the RPE during the fourth day. A definitive elastic layer is present during the ninth day and becomes more apparent following subsequent stages of development. An outer collagenous layer begins development during the tenth and twelfth days. Collagenous fibrils average 37 nm in diameter and display axial periodicity measuring 46 nm between major periods. These measurements increase with age as do the number of collagenous fibrils. Only isolated patches of basal lamina are observed in association with the choriocapillary endothelium by the twentieth day.

Key words: Bruch's membrane, development, TEM, basal lamina, amorphous material, microfibrils, collagenous fibrils, elastic fibers

Bruch’s membrane is a characteristic component of the choroid in the vertebrate eye. The fine structure of Bruch’s membrane is well established in normal and aging adult tissues, appearing as a layer of various fibrous connective tissues. The thickness of this “membrane” varies with age and species; however, its characteristic fine structure prevails in all vertebrates studied to date. Bruch’s membrane typically consists of five layers, including (1) the basal lamina of the retinal pigment epithelium (RPE), (2) an inner collagenous layer, (3) an elastic layer, (4) an outer collagenous layer, and (5) the basal lamina associated with the endothelium of the choriocapillaris. Bruch’s membrane undergoes numerous structural changes in man after 30 years of age and with the occur-

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rence of senile maculopathies. These changes include an increased deposition of fibrous connective tissues.\(^8\)\(^9\)

In contrast, little attention has been given to the development of Bruch's membrane as observed by transmission electron microscopy (TEM). Previous studies concentrate primarily on developing tissues associated with Bruch's membrane.\(^10\)\(^-\)\(^13\) Takei and Ozanics\(^14\) and Lerche\(^15\) offer more definitive studies on the development of Bruch's membrane in the monkey and man. This investigation will present a detailed TEM account of the normal development of Bruch's membrane in the chick from the time of early basal lamina formation to the synthesis of microfibrils, unit collagenous fibrils, and elastic fibers.

**Materials and methods**

Fertile pullet eggs (White Leghorn strain) were incubated at constant humidity at 38°C for periods ranging from 2 to 21 days (hatching). Upon removal from the egg, chick embryos were immersed in cacodylate-buffered glutaraldehyde-paraformaldehyde fixative (pH 7.4)\(^16\) and staged according to the Hamburger-Hamilton series.\(^17\) While in aldehyde fixative, the eyes were excised, and the anterior portion and vitreous body were removed to facilitate optimal fixation. Small pieces of tissue, including the choroid and retina, were obtained from the posterior portion of each eye. Specimens remained in fixative from 1 to 3 hr, followed by a wash in 0.2N cacodylate buffer and post-fixation in 2% osmium tetroxide\(^16\) for 1 hr. Following a wash in 0.144N cacodylate buffer, all tissues were dehydrated in ascending grades of ethanol followed by propylene oxide. Each specimen was then embedded in Epon 812\(^18\) and oriented for cross-sectional microtomy. Thick and thin sections were cut on a Sorvall Porter-Blum MT-2B ultramicrotome. Thick sections were stained with toluidine blue for light microscopic identification. Thin sections were mounted on copper grids and stained with uranyl acetate\(^19\) for 1 hr and lead citrate\(^20\) for 15 min. All specimens

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**Fig. 1.** Outer layer of optic cup (2½ days, stage 17). A continuous basal lamina (BL) is associated with the periphery of the optic cup (OC). Large intercellular spaces (large arrows) are conspicuous between the cells. Free mesenchymal cells (MC) and a sparse population of fibrous connective tissues (small arrows) are present within the surrounding tissue space (TSP). (×8000.)
Fig. 2. Optic cup and surrounding tissue space (2½ days, stage 17). Numerous microfibrils (MF) measuring 17 nm in diameter are present within the tissue space (TSP). Note the flocculent, amorphous material (arrows) associated with many of the microfibrils and the basal lamina (BL) at the external surface of the optic cup (OC). (×34,000.)

Fig. 3. Appearance of collagenous layer within the presumptive Bruch’s membrane (5 days, stage 26). Small collagenous fibrils (CF) are now present at the periphery of the retinal pigment epithelium (RPE). These fibrils average 29 nm in diameter. A demonstrable periodicity in these fibrils is difficult to discern. (×38,000.)
Fig. 4. Appearance of elastic layer within Bruch's membrane (9 days, stage 35). A discontinuous layer of elastic fibers (EL) is now forming between the retinal pigment epithelium (RPE) and the endothelium (E) of the choriocapillaris. (x 13,000.)

Fig. 5. Definitive inner collagenous and elastic layers (10 days, stage 36). The amorphous and microfibrillar (arrows) components of the elastic layer (EL) are conspicuous at this stage. The inner collagenous layer (ICL) is now more extensive. Note the prominent basal infoldings of the retinal pigment epithelium (RPE). An endothelial cell (E) of the choriocapillaris is illustrated at the upper right of the micrograph. (x 30,000.)
were examined in a JEM-100B transmission electron microscope.

**Results**

The cells of the outer layer of the optic cup were loosely arranged with many spaces between adjacent cells following 2½ days of development (Fig. 1). The only component of Bruch's membrane which was present was a continuous basal lamina associated with the basal surface of the presumptive RPE. The surrounding tissue space at the periphery of the optic cup contained free mesenchymal cells and associated extracellular fibrous connective tissues. These fibrils measured 17 nm in diameter, a size characteristic of "microfibrils" (Fig. 2). A flocculent, amorphous material often adhered to the surfaces of these microfibrils.

During the fifth day, an intermittent layer of connective tissue fibrils was positioned adjacent to the basal lamina of the differentiated...
RPE (Fig. 3). These fibrils measured 29 nm in diameter and fell within the size range of early collagenous fibrils. They were present where the choriocapillaris became established and in intercapillary regions. These fibrils designate the inner collagenous layer of Bruch’s membrane.

Another definitive layer of fibrous connective tissues was apparent during the ninth day (Fig. 4) and became more conspicuous by the tenth day (Fig. 5). This layer represents the elastic component of Bruch’s membrane, containing randomly arranged microfibrils embedded in a varying electron dense matrix.

A fourth component, the outer collagenous layer of Bruch’s membrane, appeared during the tenth and twelfth days. It consisted of collagenous fibrils positioned between the elastic layer and the choriocapillaris (Fig. 6). The inner collagenous layer was more extensive than in previous stages. Collagenous fibrils averaged 37 nm in diameter and possessed a measurable axial periodicity of 46 nm (Fig. 6, inset). Frequently, amorphous material was associated with their surfaces. The elastic layer remained discontinuous and contained microfibrils which measured 15 nm in diameter.

From the sixteenth to eighteenth days the collagenous fibrils increased in number, particularly within the inner collagenous layer (Fig. 7). They measured from 39 nm (16 days) to 43 nm (18 days) in diameter and possessed an axial periodicity of approximately 65 nm (Fig. 7, inset). Both the inner and outer collagenous layers were noticeably more extensive by the twentieth day (Fig. 8). The fenestrated elastic layer was now further removed from the en-
Fig. 8. Mature Bruch’s membrane (20 days, stage 45). Bruch’s membrane now consists of five layers, including (1) the basal lamina of the retinal pigment epithelium (RPE), (2) an inner collagenous layer, (3) an elastic layer, (4) an outer collagenous layer, and (5) an indistinct basal lamina associated with the endothelium (E) of the choriocapillaris. Note the increased number of microfibrils (Inset, arrows) within the elastic layer. The area within brackets represents the inset. (×23,000; Inset, ×42,000.)

Discussion

The structural integrity of Bruch’s membrane is dependent on the deposition of extracellular fibrous connective tissues. The orderly appearance of these various elements within Bruch’s membrane correlates significantly with their sequential development elsewhere within the chick embryo.

Basal laminae represent the earliest expression of fibrous connective tissues during embryogenesis. The first to appear in the chick is that associated with the basal surface of the epiblast. This structure appears as a tangle of “primary fibrils”, which average 4 nm in diameter. The present study reveals the early establishment of a continuous basal lamina at the periphery of the optic cup, which represents the first-appearing component of Bruch’s membrane. Isolated patches of basal lamina are also associated with the choriocapillaris endothelium during late stages of development. Banerjee et al. show that basal laminae lend morphological stability to developing epithelia. Recent in vitro
studies reveal that embryonic epithelia, including the RPE, actually synthesize the constituents of their associated basal laminae. When isolated from various tissue sources, they are known to be heterogeneous in terms of their thickness and structure; however, they are chemically similar, containing collagen, other glycoproteins, and glycosaminoglycans.

Microfibrils measuring 17 nm in diameter are present adjacent to the basal lamina of the presumptive RPE following days of development. Takei and Ozanics also noted microfibrils (~18 nm in diameter) in the monkey. The source of many of these fibrils is probably the RPE. This is supported by in vitro studies which indicate that microfibrils are often produced by the epithelia with which they are closely associated. They may appear as single fibrils, doublets, or irregular bundles when observed in semithick sections with the high voltage electron microscope. Microfibrils measuring 4 to 15 nm in diameter first appear in the chick embryo during the second day of development, associated with the basal lamina of the ectoderm. They can attain a size of 20 nm in diameter and occasionally possess an irregular pattern of axial periodicity. The amino acid composition of these larger fibrils is unknown; however, evidence suggests that they are collagenous, since they are digested with collagenase and their synthesis is inhibited when proline analogues are administered to embryos. During in vitro fibrillogenesis, Trelstad et al. note that fibrillar aggregates measuring 5 nm in diameter may represent microfibrils which are "subassemblies" or "morphogenetic intermediates" in the formation of unit collagenous fibrils. Through interpretation of x-ray diffraction patterns, Miller and Parry suggest that unit collagenous fibrils consist of "supercoiled and tightly packed" bundles of microfibrils. In addition, a flocculent amorphous material is frequently observed in association with the surfaces of microfibrils. This occurs within Bruch’s membrane as well as the perinotochordal region of the chick. Frederickson et al. note that this material is abundant during microfibril growth and is digested with collagenase. They suggest that it represents a form of "molecular collagen" which is incorporated into developing fibrils. All these factors have led some investigators to support the hypothesis that microfibrils represent the structural precursor of unit collagenous fibrils. This view supports fibrillogenesis within Bruch’s membrane, since microfibrils always precede collagenous fibrils possessing axial periodicity.

Unit collagenous fibrils represent the predominant fibrous connective tissue within Bruch’s membrane. Their source was previously considered to be solely cells of mesenchymal origin, including fibroblasts and smooth muscle cells. Recent observations, however, indicate that numerous developing epithelia, including the RPE, can synthesize procollagen in vitro. Consequently, the RPE is likely a significant contributor to procollagen secretion in vivo. Unit collagenous fibrils within Bruch’s membrane increase in diameter with age. This growth trend is also noted during in vitro fibrillogenesis by the RPE and in vivo within the cervical perivertebral connective tissue of the chick. Measurable spacing between major periods within unit collagenous fibrils in Bruch’s membrane also increases with age from approximately 46 nm (12 days) to 65 nm (18 days). However, this may represent size variation rather than a growth phenomenon, as a result of fixation in glutaraldehyde and osmium and embedment in epoxy resins. Under these conditions, Hay and Dodson reported a size range of 50 to 60 nm, figures closely paralleling those of the present study. The unit collagenous fibrils within Bruch’s membrane are often associated with an electron-dense amorphous substance. Since this occurs during fibril maturation and it is known that this material is digested with collagenase, it is suspected that the amorphous substance provides molecular collagen to developing fibrils. This amorphous material may be responsible in part for enlargement of both microfibrils and unit collagenous fibrils.
opment. It remains a discontinuous layer which is located nearest to the choriocapillaris, thus demarcating an extensive inner collagenous layer and a thin outer collagenous layer. Elastic fibers typically consist of two distinct components, including microfibrils and an amorphous component of varying electron density which is known to be elastin. Microfibrils within the elastic layer of Bruch’s membrane are more numerous during subsequent stages of development. They measure from 15 nm in diameter (10 to 12 days) to 20 nm (20 days) and appear hollow in cross-section. Microfibrils which characterize elastic fibers are usually 11 to 12 nm in diameter and possess an amino acid composition which is significantly different from collagen or elastin, including the absence of hydroxyproline and hydroxylysine. This chemical difference is further supported by the fact that microfibrils of this size are not digested with either collagenase or elastase. In contrast, larger microfibrils (~18 to 20 nm in diameter) are known to be collagenous. Since the elastin-associated microfibrils observed in the present study are of the large variety, they may contain a collagenous moiety.

Bruch’s membrane certainly offers a region of intense fibrillogenesis, with the production of all categories of fibrous connective tissues. In numerous studies, including the present one, basal laminae and microfibrils make an early appearance in association with developing epithelia. Hay suggests that the basal laminae associated with the embryonic epiblast and hypoblast may function as a “railroad track” for migration of the primitive streak mesoderm. Similarly, the basal lamina associated with the presumptive RPE may act as a substratum for the migration of mesenchymal cells in the formation of the adjacent choriocapillaris. In addition, unit collagenous fibrils are not present within Bruch’s membrane until prior appearance of a basal lamina and microfibrils at the basal surface of the RPE. This phenomenon is known to occur in association with various developing epithelia in tissue culture, including the RPE. Once unit collagenous fibrils dominate the connective tissue space, the number of non-elastin-associated microfibrils and the amount of amorphous material are vastly diminished. Considering these factors, the importance of microfibrils to unit collagenous fibril and elastic fiber formation is certainly substantiated by fibrillogenesis within Bruch’s membrane. In this study, the discrepancy in the size of microfibrils within the elastic layer when compared to those of elastic fibers in other tissues is not answerable at this time. In addition, the extent to which both the RPE and nearby fibroblasts contribute to fibrillogenesis within Bruch’s membrane is yet unknown.

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REFERENCES


