Developmental Acquisition of Basement Membrane Heterogeneity: Type IV Collagen in the Avian Lens Capsule

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ABSTRACT To investigate potential heterogeneity and developmental changes in basement membranes during embryogenesis, we performed immunohistochemical analyses on lens capsules in chicken embryos of different ages using domain-specific monoclonal antibodies against type IV collagen. We found that the capsule of the newly formed lens stained uniformly with antibodies against this component of basement membranes, but with increasing age and differentiation of the lens cells the anterior lens capsule remained brightly fluorescent while staining of the posterior capsule became relatively much less intense. This antero-posterior gradient of anti-type IV collagen antibody reactivity demonstrated that developmentally-regulated changes can occur within a single, continuous basement membrane.

Basement membranes comprise a class of extracellular matrices (1) that separate epithelia from mesenchyme and envelop certain cell types such as muscle and fat cells. In some cases they are known to have specific functions, such as renal filtration by the glomerular basement membrane (2). Basement membranes may also serve as specific substrata for cell migration and organization during wound healing and regeneration, and as mediators of the epithelial-mesenchymal cell interactions that occur during the development of many organs (3–7). If indeed basement membranes do perform different, tissue-specific functions, and possibly have "informative" roles in developmental processes, they should be heterogeneous, either in their molecular composition or in the way in which their components are assembled. Alternatively, if they simply function as a general scaffolding for tissues, or as a passive framework for cell attachment, their composition and structure need not differ.

We have previously reported the production of monoclonal antibodies specific for each of three discrete, spatially separated molecular domains within chicken type IV (basement membrane) collagen (8, 9). In immunohistochemical analyses, all three react with the basement membranes of most tissues in a continuous, linear pattern. In the kidney, for example, they appear to react with all of the basement membranes available, including those of the glomerular tuft, Bowman's capsule, the renal tubules, and collecting ducts (8). In some tissues, however, these antibodies appear to stain some basement membranes very weakly, if at all. In the cornea, for example, staining of the epithelial basement membrane by each of these anti-type IV collagen antibodies is largely, if not completely, absent.

In the present investigation we report a clear-cut spatial heterogeneity within the chicken lens capsule, a continuous basement membrane that surrounds the lens, revealed by the domain-specific antibodies against type IV collagen. We have also found that this spatial heterogeneity is developmentally regulated, being undetectable in lens capsules of early embryos, and appearing at a characteristic time during development.

MATERIALS AND METHODS

Preparation of Antibodies: The production and characterization of monoclonal antibodies against three different regions of pepsin-extracted chick type IV collagen have been described elsewhere (8, 9). Inhibition enzyme-linked immunosorbent assay[1] (ELISA) with collagen types I–V, and purified fractions of three different molecular regions of type IV collagen (termed 7S, $(F1)_2F2$, and F3; see 10, 11) showed that (*a*) none of the monoclonal antibodies against type IV collagen bound to any of the other collagens (types I, II, III, or V), and (*b*) each specifically bound to only one of the molecular regions of chick type IV collagen. In the present investigation, three antitype IV collagen monoclonal antibodies, termed IV-IA8, IV-IIB12, and IV-ID2, were used. They specifically recognize the $(F1)_2F2$, F3, and 7S region of type IV collagen, respectively (8, 9).

The production and characterization of rabbit polyclonal antibodies against chick type IV collagen has been described previously (11). Just before use in immunofluorescence histochemistry, these antibodies were repurified by chromatography on a type IV affinity column.

[1] *Abbreviations used in this paper:* ELISA, enzyme-linked immunosorbent assay.

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Immunofluorescence Observations: Whole embryos of 4 to 7 d of development, and anterior eyes from 8 to 19-d-old chicken embryos and 1-d-old posthatched chicks were quickly frozen in liquid freon cooled in dry ice. These tissues were embedded in Tissue Tek OCT (Lab-Tek Products, Naperville, IL) compound and sectioned transversally through the cornea, perpendicular to the equatorial plane of the eye; 8 μm-sections were mounted on 12-spot glass slides and stained with antibody as described previously (8, 9, 11). In all experiments, staining was done with antibody-containing medium from cultures of cloned hybridomas, or with affinity-purified antibodies from immunized rabbits. Observations were usually done without prior knowledge of the antibody used. Photographs were taken with Kodak Tri-X film rated at ASA 1600, and developed in Diafine developer.

As a positive control for immunofluorescent staining of the entire lens capsule we used a monoclonal antibody against native lens capsule from adult chicks (a group 2 antibody; see 12). The production, partial characterization, and immunofluorescent staining properties of this and other antilens capsule antibodies have been described previously (12). The antigen against which this antibody is directed has not yet been identified. We do know that it does not recognize collagen types I, II, III, IV, or V, as determined by ELISA and by indirect immunofluorescent staining of collagen dried onto glass slides.

Negative controls were treated and photographed in the same way, except that the primary antibody used was a high-titer IgG monoclonal antibody directed against type V collagen. This antibody does not stain the lens capsule. The production, characterization, and staining properties of this anti-type V collagen antibody are described elsewhere (13). The lack of lens capsule staining by our antibodies against type V collagen confirms our previously reported observations (13) that these antibodies stain dense connective tissue matrices, but not basement membranes. For each antibody used, the pattern of fluorescence reported for each stage of development was derived from at least six separate experiments with tissue taken from at least two different embryos.

Enzyme Pretreatments: Before being stained with antibodies, some sections of anterior eyes from 8–19-d-old chicken embryos and 1-d-old posthatching chicks were incubated with enzymes that digest matrix macromolecules. The treatments included: (*a*) pepsin (Sigma Chemical Co., St. Louis, MO and Calbiochem-Behring Corp., San Diego, CA; 0.1 mg/ml), in 0.5 M HAc, 0.5 h, 22°C; (*b*) trypsin (Sigma Chemical Co., types III and XI, 0.1 mg/ml), in PBS, pH 7.3, 0.5 h, 37°C; (*c*) elastase (Sigma Chemical Co., type I, 0.1 U/ml and 0.05 U/ml), in PBS, pH 8.0, 0.5 h, 37°C; (*d*) testicular hyaluronidase (Sigma Chemical Co., types I-S, III, and IV, 3,000 U/ml), in 0.1 M NaAc, 4 mM $CaCl_2$, pH 4.7, 0.5 h, 37°C; (*e*) chondroitinase ABC (Sigma Chemical Co., 1 U/ml), in PBS, pH 7.3, and 0.01 M Tris, pH 7.5, 0.5 h and 4 h, 37°C; (*f*) heparinase, with heparitinase activity (Miles Laboratories, Inc., Elkart, IN; 50 μg/ml, 420 μg/ml, and 1 mg/ml), in 0.1 M NaAc, 4 mM $CaCl_2$, 1 h and 4 h, 37°C.

RESULTS

We initially observed that in unfixed cryostat sections of eyes from 17- to 19-d-old embryos and 1-d posthatching chicks, all of the antitype IV collagen antibodies brightly stained the anterior lens capsule and the equatorial zone, but reacted only slightly with the posterior capsule (Figs. 4, *a* and *b*). Conversely, an antibody against a presently undefined noncollagenous component of basement membrane, used as a positive control, stained the entire capsule (Fig. 4*c*). To determine whether the difference in antitype IV collagen staining was the result of antigenic masking of type IV by other components of the basement membrane, sections of eyes were pretreated by digestions with a number of different enzymes, including testicular hyaluronidase, chondroitinase ABC, heparinase (with contaminating heparatinase activity), trypsin, pepsin, and elastase (see Materials and Methods). None of these pretreatments measurably altered the antero-posterior gradient of staining with any of the antitype IV collagen antibodies.

Another possibility was that the gradient might result from a failure of the monoclonal antibodies to recognize some subclasses of type IV collagen, if such exist. Such hypothetical subclasses might predominate in the posterior lens capsule. To determine whether this is the case, sections of 17-d anterior eyes were stained with affinity-purified polyclonal antibodies (11) against a similar preparation of chick type IV collagen. These antibodies produced an antero-posterior gradient of fluorescence (not shown) that was very similar to, but not quite so pronounced as, that elicited by the monoclonal antibodies.

We asked whether the gradient of staining by the antitype IV collagen antibodies is already established shortly after the time of lens formation from the embryonic ectoderm, or whether it develops later, possibly in concert with the differentiation of the fiber cells in the posterior portion of the lens. We found that tissue sections of 4- to 5-d-old lenses showed uniform staining with all of the antitype IV collagen antibodies (Fig. 1*a*), as well as with the antibasement membrane antibodies (Fig. 1*b*). By 7–8 d of embryonic development, however, a gradient of antibody reactivity was evident (Fig. 2*a*), and by 11–12 d of development (Fig. 3*a*), it was essentially established. At many stages of development, the brightest capsular staining was actually at the equatorial zone, the region where lens fiber cell elongation is initiated, and where the suspensory zonula fibers attach (see for example, Fig. 2*a*). The remainder of the anterior capsule, however, always stained with a much greater intensity than did the posterior region. In contrast, the antibody used as a positive control stained the entire antero-posterior circumference of the capsule throughout development (Figs. 1*b*, 2*b*, 3*b*, and 4*c*).

DISCUSSION

The striking spatial difference in staining of the anterior and posterior regions of the lens capsule by antibodies against type IV collagen provides a clear demonstration of heterogeneity within a single, continuous basement membrane. In this case, the heterogeneity was generated during development by progressive changes in regional composition of the lens capsule, and/or the subsequent assembly of its components. The acquisition of this spatial heterogeneity may be related to one or more of the sequential developmental events that characterize the development of the avian lens (14, 15), such as the onset of synthesis of certain β-crystallins after 6 d of development (16). The linkage between lens fiber cell development and basement membrane differentiation should be testable using preparations in which the polarity of the lens has been surgically reversed in situ (17) early in development.

The precise biochemical nature of this gradient of antibody reactivity remains to be elucidated, and at least three possible explanations exist. One is that it was generated by the de novo synthesis and deposition into the posterior lens capsule of a different basement membrane collagen, with the concomitant degradation of the original type IV collagen. Consistent with this possibility is the recent preliminary report by Johnson and Beebe (18 and personal communication) on lens capsule biosynthesis in 12- to 20-d-old chick embryos which suggests: (*a*) that the epithelial cells in the anterior lens and the fiber cells in the posterior both synthesize collagenous proteins, and (*b*) the collagenous proteins extracted from the anterior lens capsule appear to differ from those of the posterior lens capsule when analyzed on SDS gels.

Another explanation for the heterogeneity is that the antigenic determinants recognized by our antibodies were present throughout the lens capsule but differentially masked in the posterior region. Masking might result from the regional deposition into the posterior capsule of other basement membrane components, or by the arrangement of type IV molecules into a different supramolecular structure in which the

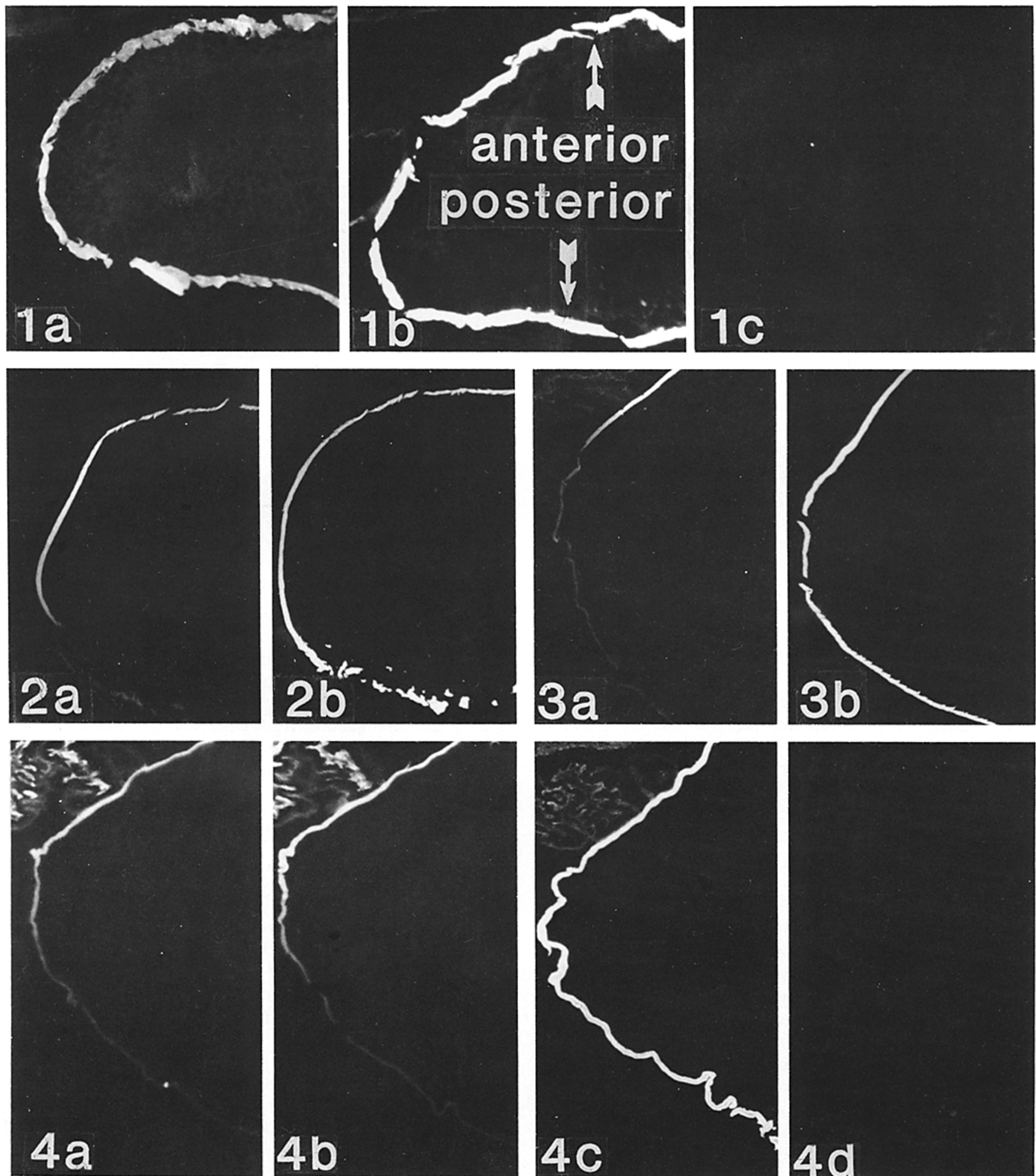

FIGURE 1–4 Indirect immunofluorescence micrographs illustrating staining patterns in sections of lens capsules from chickens at different stages of development, obtained with a monoclonal antibody directed either against one of the type IV collagen domains (Figs. 1 *a*, 2 *a*, 3 *a*, and 4, *a* and *b*), or, as a positive control, against a noncollagenous basement membrane component (Figs. 1 *b*, 2 *b*, 3 *b*, and 4 *c*). Negative controls were stained with an antibody against type V collagen (Figs. 1 *c* and 4 *d*). Figs. 1 *a* and 4 *a* are stained with IV-IA8; Figs. 2 *a* and 3 *a* with IV-IIB12; and Fig. 4 *b* with IV-ID2. The ages of the embryos are the following: Fig. 1, 4 d; Fig. 2, 8 d; Fig. 3, 11 d; and Fig. 4, 1 d posthatching. In all figures the anterior-posterior orientation of the lens is as shown in Fig. 1 *b*. The staining seen in the upper left corner of Figs. 4, *a–c* is in the basement membrane of a portion of the adjacent ciliary body. × 118 (Fig. 1); × 47 (Figs. 2–4).

determinants are sterically unavailable to antibody. If type IV collagen is indeed masked in the posterior lens capsule, then such masking would likely involve the entire molecule, since both inhibition ELISA (8, 9) and rotary-shadowing preparations (Mayne et al., unpublished results) showed that the three antibodies we employed bound to widely separated domains of the type IV molecule. Our attempts to effect unmasking gave negative results, but unknown types of antigenic masking, and methods to achieve unmasking, remain a distinct possibility. This is underscored by our recent observations that determinants in type V collagen can be completely masked in tissues; unmasking these determinants requires pretreatment of the sections with agents that produce swelling of the collagen fibrils (13). A definitive answer to this question will require further biochemical and immunochemical analyses comparing anterior and posterior lens capsules.

A third possibility is selective dilution and/or degradation of the type IV collagen in the posterior lens capsule due to rapid growth in this region. In a constantly expanding structure such as the growing embryonic lens, how is new material added to effect an expansion in the morphologically continuous capsule? Between 4 and 8 d of embryonic development, the time interval during which the antero-posterior differential in type IV collagen reactivity forms, the diameter of the eye increases three fold (19, 20); the corresponding increase in the surface area of the lens is more than fourfold (Johnson and Beebe, personal communication). Morphometric analyses of growing embryonic chicken lenses (21, 22) suggest that the major portion of this expansion probably occurs in the posterior region. Since biosynthetic (18) and radioautographic (23, 24) evidence suggests that both the anterior lens epithelial cells and the posterior fiber cells secrete capsular material, the loss of type IV collagen reactivity in the posterior capsule might be due not to its replacement by a different collagen, but to its rapid dilution by stochastically inserted new non-collagenous components. Whereas such a process would not necessarily require concomitant degradation of type IV collagen, the dissolution of the network of basement membrane collagen in the posterior lens capsule might well facilitate the rapid expansion of this region.

The results presented here also indicate that growth of the lens capsule by the deposition of new basement membrane lamellae to its inner surface, as may occur in the adult (23, 24), is unlikely. Otherwise, in older embryos the posterior capsule, when stained with the antibodies, would have a bilaminar appearance characterized by an inner unstained region of new material surrounded by an outer fluorescent layer of material deposited before 8 d of development. Moreover, such a pattern of deposition would not result in net expansion of capsular surface, only a thickening.

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