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Organization of collagen types I and V in the embryonic chicken cornea: Monoclonal antibody studies

(lathyrism/collagenase/immunohistochemistry)

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ABSTRACT **To determine whether type V collagen is antigenically masked *in situ* by its fibrillar organization, two different methods were used to perturb selectively the structure of collagen fibrils in sections of embryonic chicken corneas. The experimentally modified tissues were probed by immunohistochemical procedures with monoclonal antibodies against types V and I. A lathyritic agent was used to block crosslinking of newly synthesized collagen. This results in reversible temperature-sensitive alterations in fibrillar packing, such that freshly formed collagen fibrils retain their aggregated state at 37°C but become dissociated upon cooling. Type V-specific immunofluorescence remained masked at 37°C but was revealed at 0°C. The effect of temperature was partially reversible, indicating that type V collagen is normally unavailable for antibody binding because of its fibrillar arrangement. In sections of normal corneas, treatment with corneal collagenase, which degrades type I collagen, but not type V, also unmasked the latter. This implicates type I collagen as the masking agent. We propose that collagen types I and V are incorporated together in heterotypic fibrils.**

Little is known of the factors that control the arrangement of collagen in various extracellular matrices, but it may be significant that the cornea in a variety of species is relatively rich in type V (5–20% of total collagen; see refs. 1–5). In the avian corneal stroma, collagen is organized in well-oriented lamellae of thin (25 nm), striated fibrils of uniform diameter (6). In contrast, the adjacent sclera contains a meshwork of interlaced bundles of thicker fibrils of more variable diameter (5, 7). To visualize the distribution of collagen in avian corneas and other tissues, we previously have used monoclonal antibodies against the $[\alpha 1(V)]_2\alpha 2(V)$ form of type V collagen (8, 9) and others against type I (10) for immunohistochemical analysis. In intact, unaltered sections of embryonic chicken corneas, type V was detected only in Bowman's membrane, an acellular subepithelial collagenous matrix containing thin (20-nm diameter) fibrils. Other ocular and extraocular matrices were negative. Efforts to expose this molecule by treating with a variety of proteases and glycosaminoglycan-degrading enzymes failed (8), suggesting that such noncollagenous macromolecules were not involved in masking. However, pretreating the sections with dilute acetic acid revealed a much wider distribution of type V collagen. In acid-treated sections, type V collagen, like type I, was found in stromal matrices of a variety of tissues; immunofluorescence was particularly bright throughout the cornea. Therefore, we proposed that the acid pretreatment might have acted by swelling collagen fibrils, making type V accessible to the antibody.

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In the present study, we tested the hypotheses (*i*) that type V collagen may be masked in tissues by some form of supramolecular fibrillar organization and (*ii*) that such masking might involve a close association of type V collagen with type I fibrils. This was done by selectively altering the structure of collagen fibrils in sections of avian eyes, again using the monoclonal antibodies as probes. In one method, fibril structure was reversibly altered by temperature manipulation of lathyritic (11) tissues. The application *in ovo* of the lathyrogen β-aminopropionitrile (β-APN) blocks the formation of crosslinks in newly synthesized collagen (12), thereby producing reversible temperature-sensitive alterations in intermolecular packing such that collagen fibrils retain their aggregated state at 37°C but become dissociated at 4°C (13, 14). In another approach, type I collagen was selectively degraded with vertebrate collagenase (15), which cleaves this collagen but not type V. Both methods of perturbing fibrillar structure, one reversible and the other irreversible, demonstrated by immunofluorescence the unmasking of type V collagen in the corneal stroma.

MATERIALS AND METHODS

Preparation of Tissues. Chicken embryos were made lathyritic by applying β-APN (10 mg/ml in sterile Hanks' balanced salt solution; 2.5 mg per egg per injection) onto the chorioallantoic membrane on days 13 and 16 of incubation. Fresh, unfixed anterior eyes from 17-day-old normal and lathyritic embryos were frozen in liquid freon cooled in dry ice, and cryostat sections were prepared for immunohistochemical analysis as described (8).

Antibodies. Preparation and characterization of type-specific monoclonal antibodies against collagen types V (V-DH2; see ref. 8), IV (IV-IA8; see ref. 16) and I (I_1B6; see ref. 17) used in these experiments have been described. Staining with the primary antibody was carried out by using medium from cultures of the cloned hybridomas.

Temperature Effects on Anti-Collagen Immunofluorescence. To determine the effect of temperature on the pattern of anti-type V collagen-specific immunofluorescence, slides carrying frozen sections from both normal and lathyritic embryos were immersed for 16–24 hr in Coplin jars filled with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (P_i/NaCl) at either 0°C (cooled in an ice-water bath) or 37°C. They were treated for 1 hr with V-DH2 at the experimental temperature and then washed in three changes of Ca^{2+},Mg^{2+}-free P_i/NaCl at that temperature. After an additional wash at room temperature, the sections were stained with rhodamine-conjugated second antibody and processed as described (8). To test for reversibility of the temperature effect on staining lathyritic corneas, a group of slides was immersed in Ca^{2+},Mg^{2+}-free P_i/NaCl at 0°C for 16–24 hr, transferred to a

Abbreviation: β-APN, β-aminopropionitrile.

37°C bath for an additional 16–24 hr, and then treated with antibodies at 37°C as described above. Another group was sequentially exposed overnight to 0, 37, and 0°C before staining at 0°C. All experiments included slides stained with IV-IA8 (against type IV collagen) to control for nonspecific binding of antibody to stromal matrices. These procedures were performed with sections of corneas from four or more lathyritic embryos and three normal embryos. All experiments were performed three or more times. Stained sections were examined with a Zeiss microscope equipped for epifluorescence; all photomicrographs were exposed and printed identically.

Digestion of Tissue Sections with Collagenase. Mammalian collagenase from cultures of passaged stromal fibroblasts from adult rabbit corneas was prepared and partially purified as described (18). Just before use, the latent collagenase was activated with trypsin, which was then inhibited with soybean trypsin inhibitor. The collagenolytic activity of each batch of activated collagenase (as well as aliquots to which inhibitors of collagenase were added) was monitored by radioisotopic assay (18). Its specificity for type I collagen and lack of reactivity with type V was assayed by $NaDodSO_4$/polyacrylamide gel electrophoresis of the reaction products.

To degrade type I collagen *in situ*, sections of anterior

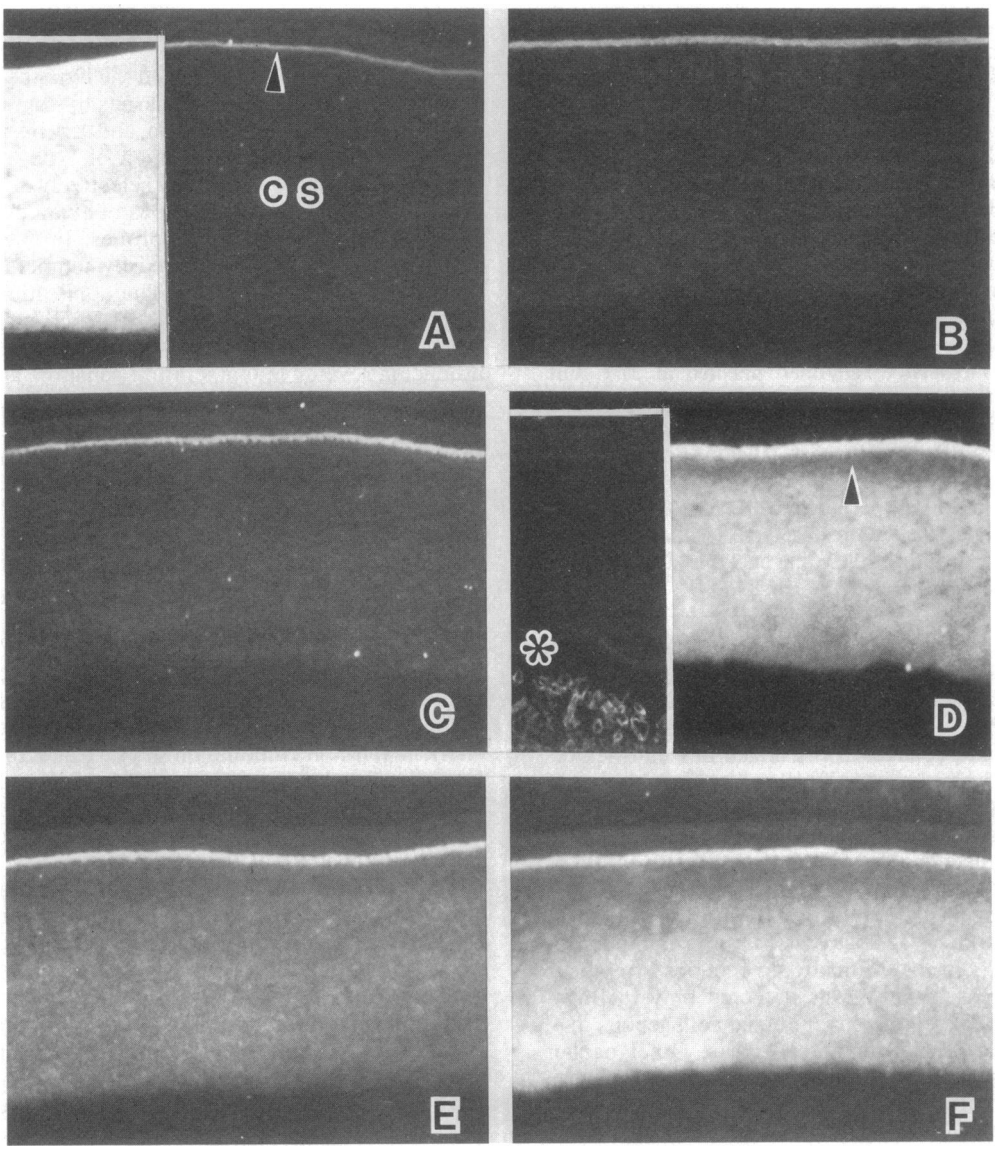

FIG. 1. Photomicrographs of corneal sections incubated at different temperatures in Ca^{2+},Mg^{2+}-free P_i/NaCl and then stained at 0°C or 37°C for type V or type IV (basement membrane) collagen. (×82.) (*A*) Normal cornea incubated and stained at 37°C for type V collagen with V-DH2. Only Bowman's membrane (arrow) is fluorescent. The underlying corneal stroma (CS) is negative. (*Inset*) Normal cornea that had been exposed to dilute HOAc to unmask type V collagen, then incubated and stained at 37°C for type V. Here, the entire stroma as well as Bowman's membrane is brightly fluorescent. (*B*) Normal cornea stained at 0°C for type V collagen, showing fluorescence only within Bowman's membrane. (*C*) Lathyritic cornea stained at 37°C for type V collagen. The pattern of fluorescence is similar to that seen in normal corneas (pictured above). (*D*) Lathyritic cornea stained at 0°C for type V collagen. Here, bright fluorescence is seen within the corneal stroma, and the intensity of staining of Bowman's membrane is enhanced. A region of the anterior stroma (arrow) is stained more faintly. (*Inset*) Lathyritic cornea stained at 0°C with antibody against type IV collagen. Within the cornea, only Descemet's membrane (asterisk) is lightly stained. The fluorescent profiles below the asterisk represent staining of blood vessels and muscle fibers of the iris. (*E*) Lathyritic cornea incubated at 0°C, then 37°C, and then stained at 37°C for type V collagen. Immunofluorescence within the stroma is noticeably less intense than that of corneas stained at 0°C (Fig. 1*D*) but has not decreased to the levels seen in corneas stained at 37°C (Fig. 1*C*). (*F*) Lathyritic cornea incubated at 0°C, 37°C, and then back to 0°C before being stained at 0°C for type V collagen. Bright fluorescence has returned to the corneal stroma.

eyes from normal 17-day-old chicken embryos were incubated with active collagenase for 2–24 hr at 37°C. Sections used as controls were incubated with the enzyme in the presence of EDTA (10 mM) or 1,10-phenanthrolene (1 mM), both inhibitors of collagenolytic activity, or with soybean trypsin inhibitor-inactivated trypsin. Sections were then washed at room temperature with Ca^{2+},Mg^{2+}-free P_i/NaCl; stained with monoclonal antibodies V-DH2, I_1B6, or IV-IA8 for 1 hr at room temperature; and processed for immunofluorescence histochemistry.

RESULTS

Temperature-Dependent Behavior of Lathyritic Corneas. Fig. 1 *A–D* shows the patterns of immunofluorescence observed when slides containing sections of normal and lathyritic corneas from 17- to 18-day-old chicken embryos were immersed overnight in neutral buffer at either 0°C or 37°C and then stained with antibody V-DH2 against type V collagen. Sections of normal corneas stained at either temperature (Fig. 1 *A* and *B*) exhibited essentially the same staining pattern, characterized by a fluorescent Bowman's membrane (arrow in Fig. 1*A*) and little or no staining of the stroma (CS).§ In contrast, a striking effect of temperature was seen in the lathyritic group. In sections of lathyritic corneas that had been preincubated and stained at 37°C (Fig. 1*C*), the pattern of fluorescence was virtually indistinguishable from that of normal corneas. At 0°C, however, bright fluorescence was present within the stroma, and Bowman's membrane exhibited enhanced staining (Fig. 1*D*). Comparatively fainter fluorescence was observed in a region of the anterior stroma (arrow in Fig. 1*D*) just beneath Bowman's membrane (see *Discussion*).

The emergence of stromal fluorescence at low temperature was not merely due to nonspecific binding of antibody by a disorganized collagenous matrix. Lathyritic corneas stained at 0°C for type IV (basement membrane) collagen (Fig. 1*D Inset*) showed fluorescence only in Descemet's membrane (asterisk). Serving as positive controls were sections unmasked with dilute HOAc as described (13) and stained at 37°C for type V collagen (Fig. 1*A Inset*). They were brightly fluorescent throughout the stroma.

The reversibility of the temperature effect on the structural organization of lathyritic collagen was investigated by transferring corneal sections from 0°C to 37°C for 16–24 hr before treatment with antibodies at 37°C (Fig. 1*E*). In these preparations, stromal fluorescence was markedly diminished compared to that of sections stained at 0°C (Fig. 1*D*) but was still noticeably brighter than that of 37°C-stained sections that had never been cooled to low temperatures (Fig. 1*C*). Sections of lathyritic corneas that were incubated sequentially at 0, 37, and back to 0°C before being stained with V-DH2 at 0°C showed restoration of bright stromal fluorescence (Fig. 1*F*).

Effect of Digestion with Corneal Collagenase. Fig. 2 *A–D* shows sections of anterior eyes from normal chicken embryos incubated for 5 hr at 37°C with active or inhibited corneal collagenase and then stained with monoclonal antibodies against collagen types V or I. Sections treated with inhibited collagenase or the trypsin–soybean trypsin inhibitor control solution and stained for type V collagen exhibited the normal masked pattern of immunofluorescence (Fig. 2*A*) in which staining was confined to Bowman's membrane of the cornea. In contrast, the distribution of type I collagen in such control sections (Fig. 2*C*) was, as reported (10), much more extensive, with bright fluorescence in Bowman's membrane and the corneal stroma as well as in the stromal matrices of the sclera and eyelid dermis.

Incubation with active collagenase, which cleaves type I collagen but not type V, produced dramatic differences in collagen-specific immunofluorescence (Fig. 2 *B* and *D*). Staining for type V collagen (Fig. 2*B*) was now very bright throughout the corneal stroma; in Bowman's membrane, the intensity of fluorescence also appeared enhanced. Bright staining was largely confined to the cornea, being considerably fainter in the sclera and dermis (not shown). Collagenase-treated sections stained for type I collagen showed a pattern of fluorescence (Fig. 2*D*) that was the inverse of that obtained for type V. Bowman's membrane and the corneal stroma, which were brightly stained in these preparations by antibody against type V collagen, were virtually devoid of type I-specific fluorescence. The only region of the cornea in which any staining remained was along the posterior border of the stroma,¶ which was faintly fluorescent in the center and more strongly stained in the periphery (arrow). In the sclera and eyelid, however, staining for type I collagen persisted but generally was less intense than in undigested sections. Collagenase digestion did not noticeably change the pattern of basement membrane fluorescence in controls stained for type IV collagen (Fig. 2*B Inset*).

DISCUSSION

Digestion with vertebrate collagenase revealed a correlation between the specific cleavage of type I collagen and unmasking of type V. This implicates type I as the masking agent. From this observation, we propose that collagen types I and V may be incorporated together within the same fibril. In support of this general concept is the recent biochemical isolation of crosslinked peptides indicating a covalent molecular association between collagen types I and III in bovine and human connective tissues (19). The mechanisms by which such structures might be assembled are unknown, but we do know from immunofluorescent staining of corneas from staged chicken embryos that type V collagen becomes masked either intracellularly or very soon after its deposition (9). It is possible that, during fibrillogenesis, type V collagen might serve as a core around which type I collagen is assembled. The resolution of the immunofluorescence method is not adequate to show the detailed relationship between type V and type I collagens; this will require immunoelectron microscopy.

The functional significance of closely associated collagen types I and V is a matter of speculation; one possibility is that the amount of type V collagen in extracellular matrices may be involved in regulating fibril diameter. Biochemical measurements of type V have shown that the cornea, which contains collagen fibrils of uniformly small diameter, is relatively rich in type V collagen. In addition, the smallest collagen fibrils in the cornea are those found in Bowman's membrane (1), where type V masking is incomplete. It is possible that a comparatively higher proportion of type V collagen within the fibrils of this latter structure results in exposure of the antigenic determinant on the fibrillar surface.

In lathyritic corneal stromas, the unmasking of type V collagen at low temperature indicates that the inaccessibility of the epitope to which V-DH2 binds is due to packing of the

§Although the pattern of fluorescence in normal corneas was similar at 0°C and 37°C, a small difference, in fact, was consistently observed. Close examination of corneas stained at 37°C revealed no stromal fluorescence, whereas stromas stained at 0°C had a faintly fluorescent patina. This subtle effect of temperature on stromal staining for type V collagen mirrors the more striking difference found in the lathyritic group and may be attributable to a limited degree of fibrillar swelling taking place at 0°C.

¶It was not possible to determine with certainty in these sections whether the staining of the posterior border of the stroma was within Descemet's membrane or restricted to the stromal matrix. Immunoelectron microscopic analysis will be required to clarify this question.

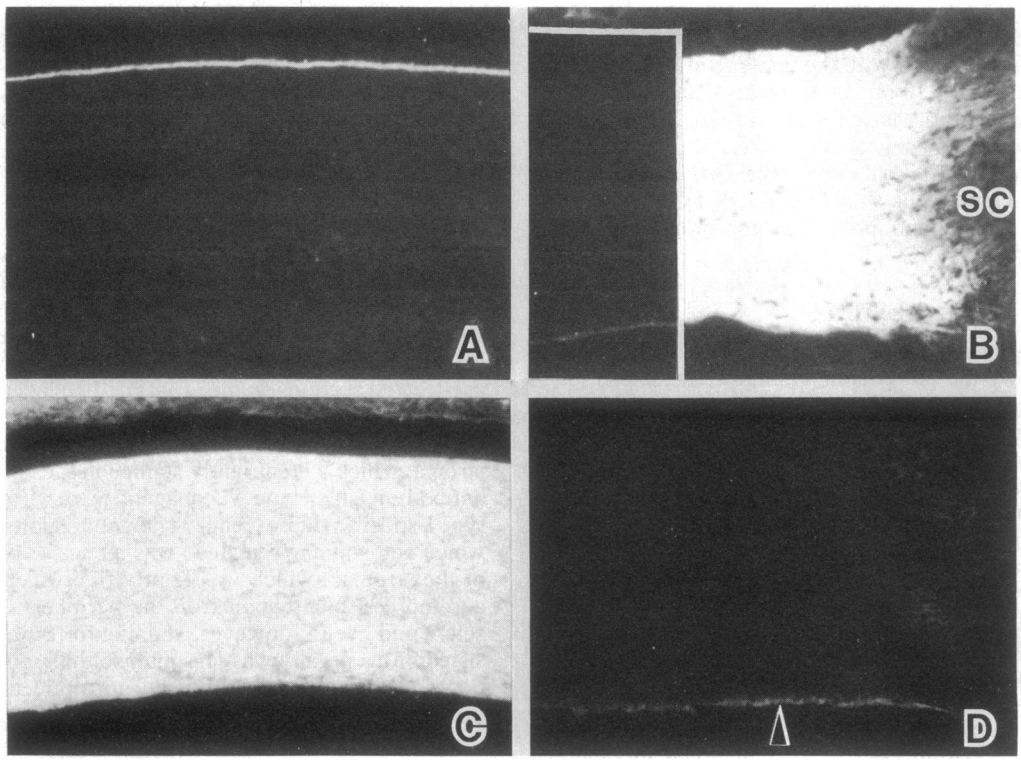

FIG. 2. Photomicrographs of sections of normal corneas incubated with active or inactivated corneal collagenase for 5 hr at 37°C and then stained with antibody against collagen types V, I, or IV. (*A* and *B*) Distribution of type V collagen in undigested and digested sections, respectively. (*C* and *D*) Type I staining in comparable sections. (×82.) (*A*) Corneal section incubated with collagenase to which EDTA had been added to inhibit collagenolytic activity and then stained for type V. Only Bowman's membrane is fluorescent. (*B*) Corneal section digested with active collagenase. Type V-specific immunofluorescence appears throughout the stroma as well as in Bowman's membrane. In the adjacent sclera (SC), the staining intensity is markedly reduced. (*Inset*) Collagenase-digested section stained for type IV (basement membrane) collagen. Here, only Descemet's membrane is fluorescent. (*C*) Corneal section incubated with collagenase plus EDTA and then stained for type I collagen. Bowman's membrane and the entire corneal stroma is brightly fluorescent. The staining at the top of the picture is that of the eyelid. (*D*) Corneal section digested with active corneal collagenase and then stained for type I collagen. Immunofluorescence has disappeared from Bowman's membrane and the posterior portion of the eyelid. Within the stroma, only a narrow band (arrow) along the posterior border remains stained. Faint staining in the adjacent sclera (right-hand side of picture) can also be seen.

collagen molecules within fibrils. This unmasking is largely but not completely reversible, as shown by the partial return of the masked pattern of immunofluorescence when the temperature of cooled tissue sections was raised again. It seems likely that most of the collagen becomes repacked into tight fibrils on warming, again blocking antibody binding. That the process is incomplete can be explained by a failure of some fibrils to reform a compact organization and/or by the extraction of some of the lathyritic type I collagen.

The reversibility of the temperature effect on type V-specific immunofluorescence makes it unlikely that type V collagen is masked by a noncollagenous component extractable in cold buffer. Such a molecule would become unavailable for remasking when slides are transferred to another vessel with warm buffer. When the rewarmed tissue is cooled a second time, the return of intense stromal fluorescence indicates the continued presence of type V collagen, confirming its reversible association with stromal collagen fibrils.

In cooled sections of lathyritic corneas, the area that becomes brightly stained for type V collagen probably represents a region in which a substantial proportion was deposited subsequent to the application of β-APN (13 days of incubation). Although this constitutes most of the stromal area in the sections of 17-day-old lathyritic corneas examined here, we did observe a region of fainter fluorescence in the anterior stroma. If such preparations are first treated with dilute HOAc before staining, type V-specific fluorescence within this region is as intense as that in the rest of the stroma (unpublished data). Therefore, it appears that the anterior stroma may be composed largely of collagen synthesized earlier in development and, hence, is relatively unaffected by the lathyrogen. These observations suggest that similar analyses of tissue from embryos treated with β-APN at different times in development and under various experimental conditions might allow mapping of the temporospatial pattern of collagen deposition during development, growth, and repair.

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