SYNTHESIS OF TYPE V COLLAGEN BY CHICK CORNEAL FIBROBLASTS IN VIVO AND IN VITRO

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1. Introduction

Transparency of the cornea depends partly on the regular size and spatial arrangement of the collagen fibrils [1,2]. For this reason the nature of the collagen in the corneal stroma has been the subject of many studies. Conflicting reports exist on the genetic types of collagen constituting the corneal matrix, which may in part be explained by species differences and by the developmental stages investigated. In all studies type I collagen was found to be the predominant collagen type [3–8] while ~20% type III collagen was reported in the bovine cornea [8,9]; this collagen type was not found in the rabbit cornea [6], in human corneal fibroblast cultures [10,11] or in the chick cornea [7,12]. Immunological evidence was presented for type II collagen in the mouse cornea [13]. Similarly, type II collagen was found in the primary corneal stroma of the embryonic chick eye by biochemical methods [5] and by immunofluorescence [7]. In the secondary stroma, type II collagen persisted until stage 42 in the anterior part of the stroma [7] but at levels too low to be detected by biochemical methods.

From several reports evidence is available that type V collagen (αA and αB chains; [14,15]) may constitute a considerable portion of the corneal collagen. Two collagen chains were described in the rabbit cornea with the electrophoretic mobility of αA and αB chains [6]. The synthesis of αA and αB chains in cultures of human fibroblasts was also reported [11,16].

Embryonic chick cornea fibroblasts were shown to synthesize considerable amounts of αA and αB chains in culture [12]. Here we present biochemical and immunological evidence for the existence of type V collagen in the corneal stroma of 14–17 day chick embryos which represents ~20% of the total collagen. The synthesis of type V collagen continues when corneal fibroblasts are taken into monolayer culture.

2. Materials and methods

2.1. *Extraction of collagen*

Corneas were removed from 14 day chick embryos with watchmaker forceps, and adherent tissue was carefully trimmed with small scalpels. Epithelium and endothelium were removed by short treatment with bacterial collagenase as in [12], and the stromae were cut in small pieces, homogenized in 0.5 M acetic acid and digested with pepsin (from swine stomach, 3 X cryst., Serva, Heidelberg) (0.3 mg/ml at pH 2.0) for 24 h at 4°C.

Pepsin was inactivated by raising to pH 8.0, and the opaque solution was dialyzed against water and dialyzed. For fractionation of types I and V collagen, the pepsin digest was brought to 4% NaCl (w/v) at pH 2.0 and stirred overnight. The precipitate was pelleted by centrifugation and dissolved in acetic acid, while the supernate was dialyzed against 0.05 M sodium phosphate buffer (pH 8.0). The precipitate which formed (containing most of the type V collagen) was dissolved in acetic acid.

2.2. *SDS–polyacrylamide gel electrophoresis*

This was done on 6% polyacrylamide-slab gels in glycine/Tris buffer (pH 8.6) [17] as in [18]. Gels were stained with Coomassie blue R and scanned with a Zeiss densitometer at 575 nm. ^{3}H Labeled gels were processed for fluorographic detection of

radioactively labeled bands as in [19]. Standard type V collagen from chick embryos was prepared as in [18].

2.3. *Cell culture*

Corneal fibroblasts from 14 day chick embryos were cultured in F12 medium containing 10% fetal calf serum as in [12]. Cultures were labeled on day 3 with 60 μCi L-[2,3-^{3}H]proline (30 Ci/mmol, New England Nuclear) for 24 h, and the collagens from medium and cell layer were treated with pepsin and processed for SDS gel electrophoresis as in [12].

2.4. *Immunofluorescence*

Preparation and specificity of guinea pig antibodies to chick type I collagen and rabbit antibodies to type V collagen have been documented in [20,21]. Frozen sections of 14 day embryonic chick corneas were stained with antibodies and counterstained with fluorescein-conjugated goat anti-immunoglobulins (Behring-Werke, Marburg) [20]. Corneal fibroblasts in culture were fixed with 70% ethanol and stained as in [12,19].

3. Results

3.1. *Collagen composition of the whole corneal stroma of 14 day chick embryos*

The corneal stroma of 14 day chick embryos was completely solubilized by pepsin within 24 h. When this material was analyzed by SDS–polyacrylamide gel electrophoresis, besides α1 (I) and α2 chains of type I collagen two bands were discernible which comigrated with standard αA and αB chains from chick embryos [18], as well as β-chains. Densitometric tracing of the Coomassie blue-stained gel (fig.1a) revealed that αA and αB chains accounted for ~20% of the total protein (fig.2). For better demonstration of αA and αB chains, the pepsin solubilized material was fractionated by precipitation with 4% NaCl at pH 2.0 [15]; a precipitate was obtained which contained the majority of type I collagen (fig.1d), while the supernate was enriched for type V collagen (fig.1c).

Immunofluorescence staining of a 14 day embryonic cornea with antibodies specific for type V collagen located this collagen in an even distribution throughout the stroma, similar to type I collagen (fig.3). In contrast to type I collagen, however, it is nearly absent from scleral tissues [7].

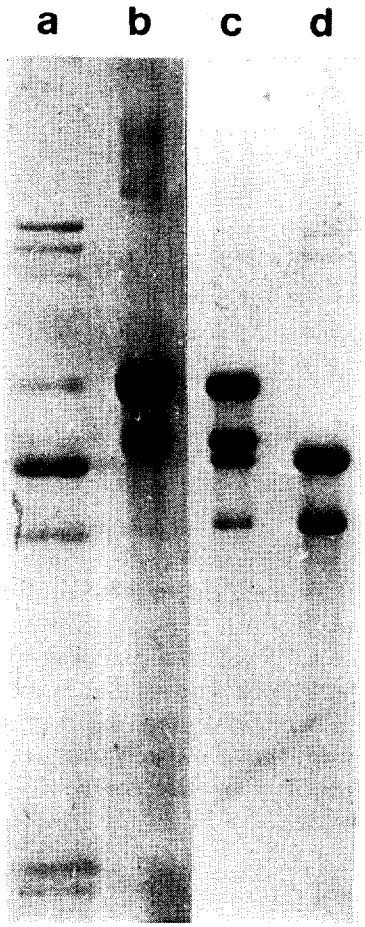

Fig.1. SDS–polyacrylamide gel electrophoresis of pepsin-extracted collagen from corneal stroma of 14 day chick embryos. Coomassie–blue stain. (a) Pepsin-digested corneal stroma. (b) Standard type V collagen from chick embryos. (c) Pepsin-digested corneal stroma, supernate of 4% NaCl fractionation. (d) Pellet obtained after precipitation with 4% NaCl.

3.2. *Type V collagen synthesis in cell culture*

Fibroblasts from the corneal stroma of 14 day chick embryos continue to synthesize types I collagen as the predominant collagen type and ~10% type V collagen when taken into monolayer culture.

We have shown the secretion of αA and αB chains into the culture medium of corneal fibroblasts by SDS gel electrophoresis [12]. Continuation of these studies demonstrated, however, that the majority of

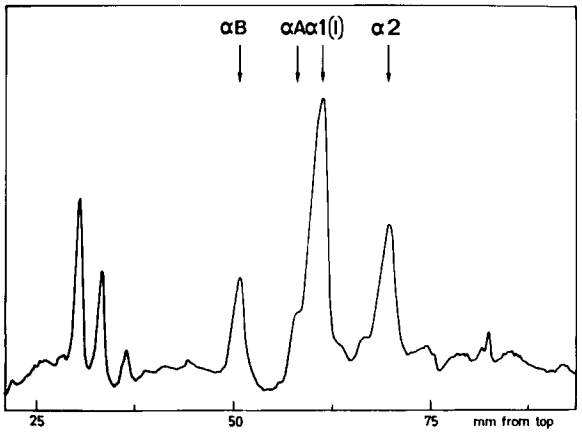

Fig.2. Densitometric scanning of fig.1a at 575 nm.

type V collagen retained in the cell layer: while type V collagen accounts for ~5% of the total collagen in the medium fraction, as determined by densitometric tracing of an SDS gel electrophoresis as shown in fig.4a, the cell layer contained ~15% type V collagen (fig.4b). When corneal fibroblasts were fixed with ethanol and stained after 3 days in monolayer culture with antibodies to type V collagen, cells revealed intracellular fluorescence similar to type I collagen (fig.5).

4. Discussion

The avian corneal stroma is of theoretical interest in collagen biochemistry as it is one of the few tissues besides bone matrix which contains type I collagen but no type III collagen [3,7]. The homogeneity in

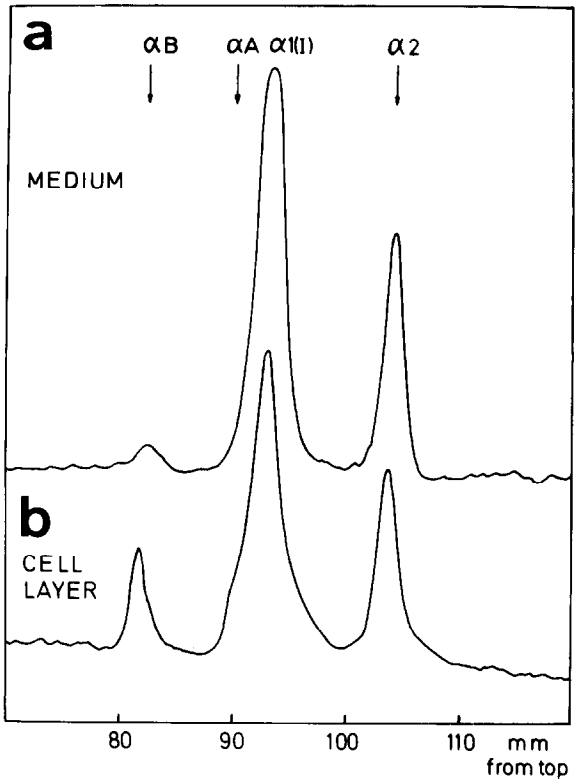

Fig.4. Collagen synthesized by corneal fibroblasts on day 7 in culture. Cultures were labeled with [^{3}H]proline for 24 h, and cell layer and medium were digested with pepsin at 15°C at pH 2. The pepsin digest was separated by SDS gel electrophoresis on 6% polyacrylamide slab gels, and the radiolabeled bands were revealed by fluorography and scanned at 470 nm.

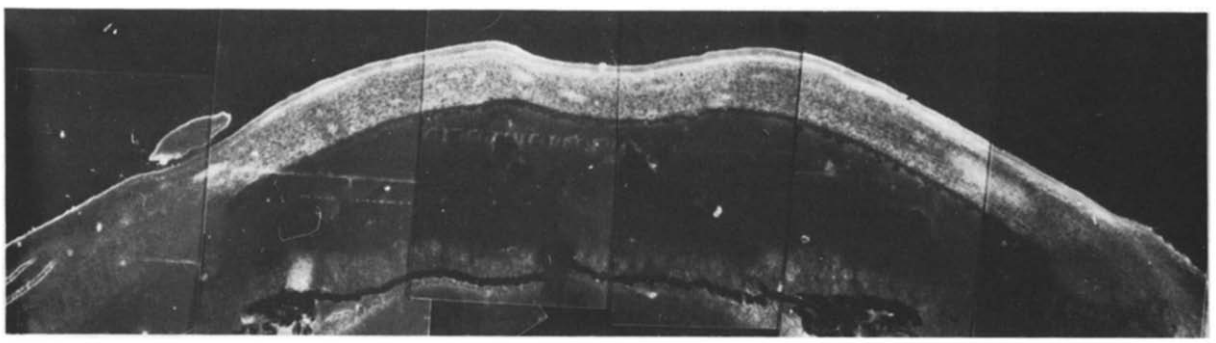

Fig.3. Immunofluorescence labeling of a 14 day embryonic chick cornea (frozen section) with rabbit antibodies to chick type V collagen, counterstained with fluorescein-conjugated goat anti-rabbit γ-globulin.

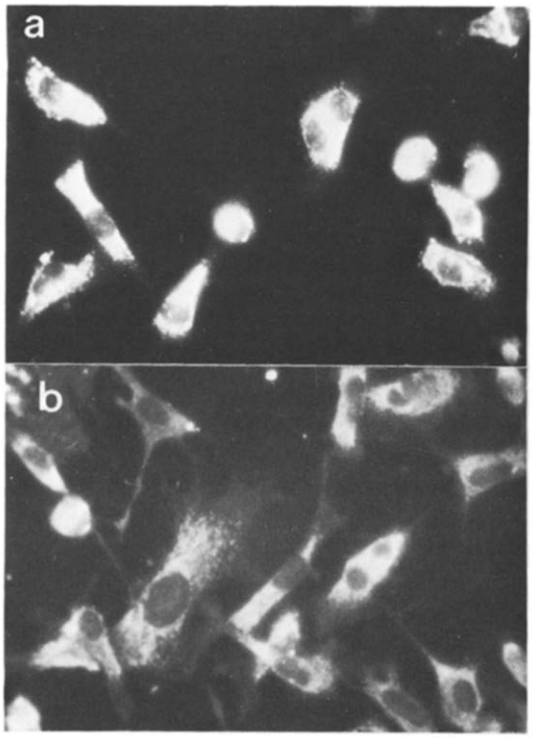

Fig.5. Immunofluorescence labeling of corneal fibroblast in monolayer culture with antibodies to type I collagen (a) and type V collagen (b).

the collagen composition was thought to be one of the reasons of the extremely regular size and spacing of the collagen fibrils in the stroma [2,22]. Here, however, immunological and biochemical data indicate that type I collagen is not the only collagenous constituent. Type V collagen accounts for almost 20% of the total collagen of the corneal stroma. Preliminary data suggest that a similar ratio of type V collagen also is present in the cornea of the adult chick (A. Schell, K. v/d M., unpublished).

Ultrastructurally, the only collagenous domain in the corneal stroma is the 25 nm diam. fibril. The question remains open whether type V collagen codistributes with type I collagen in the same collagen fibrils of the stroma, or whether it is assembled in separate fibrils of similar ultrastructural appearance as type I collagen fibrils. The possibility also exists that it is located between type I collagen fibrils as an electron microscopically unstructured entity.

It is not known whether type V collagen is capable of forming crossbanded fibrils in any tissue; in vitro it forms crossbanded collagen fibrils when dialyzed against phosphate-buffer saline, similar to types I, II or III collagen [23].

Immunofluorescence studies of the extracellular matrix formed in muscle cell cultures indicated that type V collagen may exist in fibrous elements codistributing with types I and III collagen [21]. In view of this finding it is likely that in the cornea type V collagen is incorporated in the type I collagen fibrils. Ultrastructural studies on the identification of this collagen in the corneal stroma are in progress (M. Hendrix, E. D. Hay, K. v/d M., in preparation).

Type V collagen has been identified in many tissues such as fetal membranes [14] vessel walls [15], bone and cartilage [24] and skeletal muscle [25] after extraction with pepsin. Unlike type I collagen, tissue-bound type V collagen resists completely to extraction with salt or acids, suggesting, that the tissue form of type V collagen is highly crosslinked.

As the amino acid composition of αA and αB chains resembles that of α1 (IV) chains in terms of high hydroxylysine content and low alanine [18,24], it is possible that type V collagen exists in most tissues like type IV collagen as unprocessed, highly crosslinked precursor molecule [26].

In cell culture, the majority of type V collagen remains associated with the cell layer; the immunofluorescence labeling located type V collagen mainly intracellularly. This was found not only in cultures of corneal fibroblasts, but also in cultures of chondrocytes, tendon fibroblasts and myoblasts (K. v/d M., H. Herrmann, J. Sasse, in preparation). The fraction of type V collagen secreted into the medium of neural retinal cell cultures was shown to be a high MW, but soluble precursor similar to types I or III collagen [27]; a similar observation was made with type V collagen from the medium and cell layer of myoblast cultures, (H. v/d M., in preparation) suggesting that in cell culture type V collagen remains largely as unprocessed precursor molecule.

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