Relative rates of biosynthesis of collagen type I, type V and type VI in calf cornea

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The biosynthesis of type I, type V and type VI collagens was studied by incubation of calf corneas *in vitro* with [^{3}H]proline as a marker. Pepsin-solubilized collagen types were isolated by salt fractionation and quantified by SDS/PAGE. Expressed as proportions of the total hydroxyproline solubilized, corneal stroma comprised 75% type I, 8% type V and 17% type VI collagen. The rates of [^{3}H]proline incorporation, linear up to 24 h for each collagen type, were highest for type VI collagen and lowest for type I collagen. From pulse–chase experiments, the calculated apparent half-lives for types I, V and VI collagens were 36 h, 10 h and 6 h respectively.

INTRODUCTION

The predominant collagens of mammalian corneal stroma were thought to be type I collagen, with a minor amount of type V collagen [1–3]. More recently, significant proportions of type VI collagen have been found in human and rabbit cornea [4,5]. Other collagen types are also present in small amounts and at specific localizations, i.e. types III, IV, VII and VIII [6,7]. Copolymers of type I and type V collagens have also been described in corneal collagen fibrils [8]. It was suggested that the relative ratio of type V to type I could regulate fibril diameter [8]. Furthermore, type VI collagen was also shown to be located in corneal stroma and may be associated as fine filaments between the banded collagen fibrils of type I and type V collagens [9,10]. Besides the above-mentioned data on the distribution and proportions of different collagen types in corneal stroma, only a few investigations have been performed on the relative rates of biosynthesis of each collagen type [7]. The relationships between the distribution of collagen types and structural organization of corneal stroma also indicate the need for a better understanding of the regulation of the biosynthesis of the main collagens of this highly differentiated connective tissue. We have focused our attention on the main bovine corneal collagens, namely type I, type V and type VI, in order to determine their relative rates of biosynthesis. We have developed a method of cornea labelling *in vitro* in explant cultures [11]. In the present study we confirm that bovine cornea contains a significant percentage of type VI collagen and that the relative rate of type VI collagen biosynthesis is significantly higher than those of type I and type V collagens.

MATERIALS AND METHODS

Bovine corneas were dissected from eyes freshly obtained from a local slaughterhouse. In order to study the biosynthesis of collagens by keratocytes without interference by epithelial or endothelial corneal cells, corneal stroma was carefully dissected free from epithelium and endothelium [11].

Corneas were placed in Dulbecco's modified Eagle's minimum medium with 5% (v/v) foetal-calf serum supplemented by 2 mM-glutamine, ascorbic acid (50 μg/ml), penicillin and streptomycin. The explants were maintained for various periods of time in a 37 °C incubator with a 5% CO_2/air mixture atmosphere.

Radiolabelling was performed by addition to the culture medium of L-[2,3,4,5-^{3}H]proline (sp. radioactivity 105 Ci/mmol; 50 μCi/ml; purchased from Amersham-France). Incubation was stopped after various periods of time (up to 24 h) by cooling to 4 °C. For pulse–chase experiments corneas were labelled with [^{3}H]proline for 15 h, and then incubated in fresh non-radioactive medium containing excess unlabelled proline (1%) for various chase periods up to 10 h. At the end of incubation, the corneas were removed from the medium, extensively washed in fresh ice-cold non-radioactive medium and immediately frozen in liquid N_2 and then pulverized. Powdered corneas were digested with 2 mg of pepsin/100 mg tissue wet wt. in 0.5 M-acetic acid at 4 °C for 24 h. Digests were centrifuged at 15000 *g* for 30 min and the supernatants decanted. The tissue residues were again treated with pepsin, centrifuged, and the supernatants pooled with the first extract. Undigested residues were analysed for hydroxyproline content [12].

Pepsin-soluble fractions, containing collagens, were fractionated by differential salt precipitation at 0.8 M- and 2.5 M-NaCl in 0.5 M-acetic acid.

Precipitated collagens were redissolved in 0.5 M-acetic acid, dialysed against the solvent and freeze-dried. Hydroxyproline content of each fraction was determined on samples [12].

Collagen fractions were analysed by SDS/PAGE (7.5% acrylamide gel) under reducing and non-reducing conditions [13]. Proteins were stained with Coomassie Blue, and densitometric analysis was performed as previously described [14–16]. Collagen types were quantified by using the relative band densities of each α-chain polypeptide and the amount of hydroxyproline in each precipitated fraction. For type I we added α1 and α2 chains. For type V we used α1 and α2 chains, and for type VI we summed α1, α2 and α3 polypeptides. Radioactivity incorporated in each collagen α-chain was determined as described elsewhere [14–16]. Briefly, radiolabelled collagens were analysed by SDS/PAGE, and each α-chain was cut out of the gel, hydrolysed in 6 M-HCl for 24 h at 105 °C, and then radioactivity incorporated in hydroxy[^{3}H]proline was determined [14].

Standard type I, V and VI collagens were isolated from bovine placenta [6].

Susceptibility of collagenous material to bacterial collagenase was determined in each fraction with or without prior reduction

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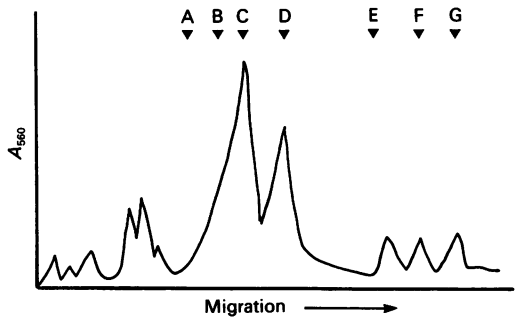

Fig. 1. Densitometric analysis of pepsin-solubilized collagen molecules precipitated at 0.8 M-NaCl and analysed by SDS/PAGE

Electrophoresis was performed under reducing conditions as described in the Materials and methods section. Gels stained with Coomassie Blue were scanned at 560 nm. Migrations are shown of (A) α1(V), (B) α2(V), (C) α1(I), (D) α2(I), (E) α1(VI), (F) α2 (VI) and (G) α3 (VI) chains.

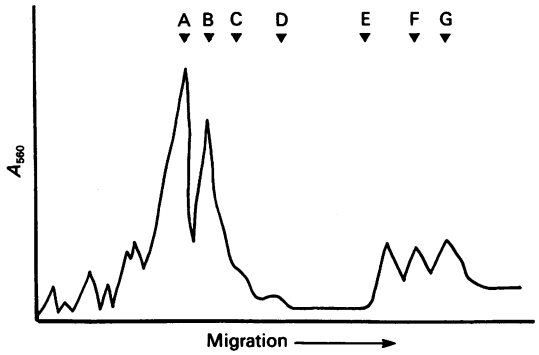

Fig. 2. Densitometric analysis of pepsin-solubilized collagen molecules precipitated at 2.5 M-NaCl and characterized as described in Fig. 1

Positions of standard α-chains are as in Fig. 1.

as in [7]. Briefly, 100 μg of collagenous material was incubated with 50 μg of *Clostridium histolyticum* collagenase (protease-free, CLSPA grade; Worthington, St. Louis, MO, U.S.A.) in 10 mM-$CaCl_2$/50 mM-Tris/HCl buffer, pH 7.5, at 37 °C for 4 h. The incubation mixture was analysed by SDS/PAGE.

RESULTS

Quantification of bovine corneal collagen types

Limited pepsin digestion solubilized up to 75% of total corneal collagens. Total pepsin extract was submitted to differential salt precipitation. The fractions obtained were analysed by SDS/PAGE under reducing and non-reducing conditions. The material precipitated at 0.8 M-NaCl represented up to 90% of pepsin-soluble collagen and showed the banding pattern of type I collagen, with appreciable amounts of type VI collagen (Fig. 1). Typically, the three α-chains of type VI collagen were detected only after reduction with β-mercaptoethanol and had apparent molecular masses estimated as 65, 50 and 45 kDa (Fig. 1). Under non-reducing conditions type VI collagen characteristic polypeptides could not be detected, but appreciable amounts of high-molecular-mass material remained in the stacking gel.

The material precipitated at 2.5 M-NaCl exhibited a characteristic profile of type V collagen with a molecular composition

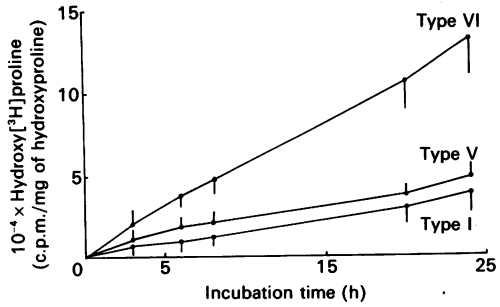

Fig. 3. Kinetics of incorporation of [^{3}H]proline into calf corneal collagen

Ordinate shows radioactivity recovered in the isolated collagen α-chains. For details see the Materials and methods section.

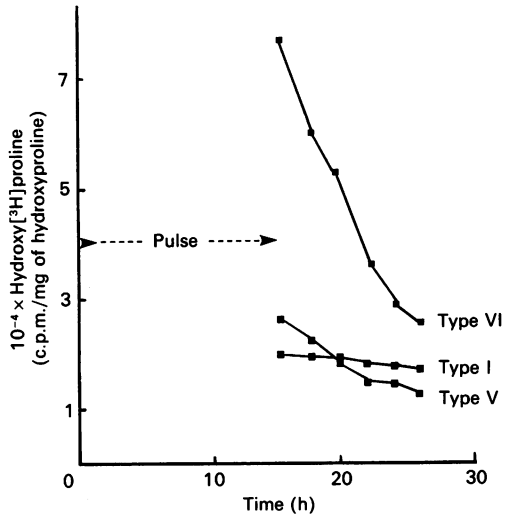

Fig. 4. Pulse–chase experiment showing the rapid turnover of collagen type VI as compared with collagens type I and type V in calf corneal stroma

[^{3}H]Proline was added to explant-cultured corneas as described in the Materials and methods section for a pulse period of 15 h, followed by a chase period up to 10 h. Incorporation of radioactivity was determined in separated collagen α-chains as described in the Materials and methods section.

of $[\alpha1(V)]_2\alpha2(V)$ and appearance of type VI collagen under reducing conditions (Fig. 2).

With prior reduction, all polypeptide α-chains were susceptible to bacterial collagenase. In contrast, without prior reduction, high-molecular-mass material in the stacking gel (probably disulphide-bonded type VI collagen) was not sensitive to collagenase (results not shown).

Densitometric analysis of these gels and hydroxyproline content of each α-chain were used to quantify the relative proportions of collagen types in cornea: 75 (±11)% for type I, 8 (±1)% for type V and 17 (±2)% for type VI.

Incubation of bovine cornea *in vitro*

Bovine corneal stroma was maintained under explant-culture conditions and labelled with [^{3}H]proline for various periods of time. Hydroxy[^{3}H]proline was determined in each collagenous α-chain separated on SDS/PAGE from pepsin-soluble fractions as described above. The incorporation of radiolabel in each collagen type was almost linear up to 24 h (Fig. 3).

Specific radioactivity of hydroxy[^{3}H]proline was significantly

higher in collagen type VI as compared with type V and type I collagens. After 24 h of labelling, the specific radioactivity of hydroxy[^{3}H]proline in type VI collagen was approx. 3.2- and 2.6-fold higher than in type I and type V collagens respectively.

To estimate the apparent turnover of each collagen type, corneas were labelled for 15 h (to obtain a sufficient amount of polypeptide-bound hydroxy[^{3}H]proline) and then incubated in fresh non-radioactive medium for various chase periods up to 10 h (Fig. 4). At each time point of the chase experiments, a similar percentage of pepsin solubilization was achieved (70–75 % of total corneal collagen).

The pulse–chase experiments indicated that the apparent turnover of type VI collagen was much faster (about 6 times) than that of type I collagen. The specific radioactivity of type VI collagen decreased to half the value obtained after the pulse period in approx. 6 h. This apparent half-life was estimated as 36 h for type I collagen and as 10 h for type V collagen.

DISCUSSION

The observations of the ubiquitous occurrence in a variety of tissues of type VI collagen are well documented (see [17] for review). However, type VI collagen represented only a few per cent of total collagens present in several tissues examined [17].

Our study confirms, however, that cornea is in this respect a special tissue particularly rich in type VI collagen [4,6,7]. In contrast with previous data indicating that type VI collagen extracted from human or rabbit cornea was precipitated between 1.2 M- and 2 M-NaCl [6,7], it appears that more than 80 % of bovine corneal type VI collagen was co-precipitated with type I collagen at 0.8 M-NaCl. Possible, as yet undefined, interactions between type I and type VI collagens in bovine corneal stroma could explain these results. According to our estimation, in calf cornea type VI collagen represents 17 % of total pepsin-solubilized collagens. Furthermore, we have obtained a relatively good yield of extraction with pepsin: up to 75 % of total corneal collagen was recovered, as estimated by hydroxyproline determination. Therefore our estimation of the percentage of the main collagen types of cornea could reflect a good approximation to the actual composition of the calf corneal stroma.

Pepsin-soluble type VI collagen in calf cornea seems to have a 1:1:1 α1(VI):α2(VI):α3(VI) chain composition. However, in its native form, type VI collagen contains large non-helical portions, representing over 50 % of its molecular mass. It seems therefore likely that the actual percentage of type VI collagen in bovine cornea could be higher than 17 % [4,6].

Radiolabelling of collagens during incubation of corneas *in vitro* in explant-culture conditions enables us to compare the relative rates of biosynthesis of the different collagen types present in cornea. Clearly the incorporation of radiolabel is much greater in type VI collagen, indicating a faster biosynthesis and turnover than for type V and type I collagens. In addition, the apparent turnover of type V collagen is higher than that of type I collagen.

The regulation of the relative proportions of the different collagen types in cornea is supposed to play an important role during its morphogenesis. The qualitative and quantitative changes detected in collagen phenotype expression in the developing cornea [8,18] are in favour of this contention.

In mature corneal stroma, the diameter of collagen fibrils seems to be related to type I: type V ratio [8]. Moreover, current concepts of corneal transparency require that small and constant distances between the collagen fibrils should be maintained [19]. Type VI collagen could occupy the space between the heterotypic fibrils composed of type I and type V collagens [10,18,20], and play the role of a stabilizing factor [21]. It seems therefore likely that the differential rates of collagen-type biosynthesis described in the present study should be related to their respective roles in the structural organization of corneal stroma.

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