Analysis of the collagen types synthesized by bovine corneal endothelial cells in culture

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Bovine corneal endothelial cells synthesize in culture predominantly type III collagen, with lesser amounts of types I and V and apparently little if any type IV. This pattern of synthesis is observed in both dividing and post-confluent cultures and irrespective of whether cells are attached to plastic or collagen-coated surfaces.

Study of endothelial cells in culture, including their biosynthetic behaviour, is important, since it could lead to a better understanding of vascular disease such as atherosclerosis or that associated with diabetes and scleroderma, where impaired endothelial function may be involved. Among the main extracellular products of these cells it might be expected that the major collagen would be type IV, that characteristic of basement membrane, and data to this effect have been presented for cells of this type from bovine aorta (Howard *et al.*, 1976; Madri *et al.*, 1980) and human umbilical vein (Jaffe *et al.*, 1976). In contrast, we have found that those from pig aorta synthesize predominantly interstitial collagens (Barnes *et al.*, 1978). Synthesis can vary from one cell batch to another, from mostly type I to largely type III, but always with only relatively low amounts of collagens types IV and V (Sankey & Barnes, 1980). Compatible with these observations, Sage *et al.* (1979) have reported that the main collagen synthesized by bovine aortic endothelial cells is type III rather than type IV.

In an attempt to understand the reason for these contrasting findings and to gain some insight into those factors controlling collagen synthesis by endothelium, we have now examined an endothelial cell type of non-vascular origin, that of bovine cornea, which produces a clearly defined basement membrane (Descemet's).

Materials and methods

Bovine eyes were obtained fresh from an abattoir. Pepsin was from Worthington Biochemical Corp.,

trypsin from Sigma Chemical Co., Dulbecco's Modified Eagle Medium (Morton, 1970) and foetal calf serum were from Gibco-Biotech, and L-[5-³H]proline was from The Radiochemical Centre. Ca- and Mg-free phosphate-buffered saline was made as described by Dulbecco & Vogt (1954).

Collagens

Type I was prepared from pig aorta (Barnes *et al.*, 1976a), and type IV from bovine anterior lens capsules essentially as described by Barnes *et al.* (1980). The purity of each collagen was established by gel electrophoresis. Carrier collagen was prepared from a pepsin digest of pig aorta (Barnes *et al.*, 1976a) as two fractions, precipitating at 0.7 M- and 1.2 M-NaCl respectively.

Isolation of bovine corneal endothelial cells

This was done under sterile conditions, based on the procedure of Slick *et al.* (1965). Briefly, after excision of the cornea, the endothelial cells on the posterior surface were detached by a short exposure (5–10 min, 21°C) to a few drops of 0.1% (w/v) trypsin in phosphate-buffered saline. Cells (from five to ten eyes) were collected by centrifugation, washed once and then seeded into a 25 cm² culture flask with growth medium containing 20% serum. When confluent, cells were subcultured and maintained in medium containing 10% serum.

Labelling of cell cultures

Cells were used for experiment after one or two subcultures and labelled just before confluence. Medium was replaced with fresh containing L-[5-³H]proline (100 µCi) and ascorbic acid (50 µg/ml). Cultures were harvested 24 h later. In some instances, cells were kept at confluence for 7 weeks before labelling.

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In other experiments, cells were grown in 60 mm culture dishes, some of which had previously been coated with either type I or type IV collagen by allowing a solution of 30 μ g in 0.1 M-acetic acid, placed in the dish, to evaporate (Kleinman *et al.*, 1979). Dishes were sterilized by u.v. irradiation before seeding. After 5 days growth, cultures just before confluence were labelled, and harvested 48 h later.

Separation of radiolabelled collagens

Cell layers and media, separately or combined, were fractionated as described by Kresina & Miller (1979). Briefly, samples were precipitated with $(\text{NH}_4)_2\text{SO}_4$ (Mayne *et al.*, 1977) after addition of carrier collagen (0.7 M-NaCl-precipitated), and the precipitate was digested with 0.01% (w/v) pepsin in 0.5 M-acetic acid for 6 h at 9°C. Interstitial collagens were precipitated with 0.7 M-NaCl. Precipitation was repeated (after addition of further carrier) and the two precipitates were combined. Collagens types IV and V were precipitated from the supernatant (after addition of 1.2 M-NaCl-precipitated carrier) with 1.2 M-NaCl. The precipitate was redissolved and type V collagen precipitated by dialysis against 0.02 M-NaCl/2 M-urea in 0.01 M-Tris/HCl, pH 8.6.

CM-cellulose chromatography and gel filtration

These were performed as described by Barnes *et al.* (1976b).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was undertaken as described by Laemmli & Favre (1973), and interrupted electrophoresis as described by Sykes *et al.* (1976). After staining, gel lanes were sectioned into 1.5 mm strips and the radioactivity in each strip was counted after solubilization in 30% H_2O_2 /0.33% NH_3 at 37°C.

Results

Cells labelled for 24 h just before confluence were harvested, cell layers and media combined and then fractionated as described in the Materials and methods section. The fraction precipitating at 0.7 M-NaCl, expected to contain interstitial collagen(s) synthesized by the cells, was examined first by CM-cellulose chromatography. A peak eluted in the position of $\alpha 1(\text{I})$ -chains of type I collagen was observed, together with a larger peak eluted in the region of $\alpha 2(\text{I})$ - and type III-derived chains (Fig. 1a). The latter peak material was examined by interrupted gel electrophoresis, which confirmed the presence of type I $\alpha 2$ -chains and γ -sized chains of type III collagen (inset to Fig. 1a). Analysis of the CM-cellulose elution profile allowed the calculation that types I and III collagens were present in the ratio

approx. 1:3. Gel filtration of the 0.7 M-NaCl-precipitated fraction indicated the presence of both γ - and α -chain-sized components in a ratio of around 3:1, thus confirming the above analysis. Final confirmation of the synthesis of collagens types I and III was achieved by examining the 0.7 M-NaCl precipitate directly by gel electrophoresis (Fig. 1b), which showed the presence of radiolabelled $\alpha 1(\text{I})$ - as well as γ -chains of type III collagen. Conversion of the γ -component into type III α -chains was shown by interrupted electrophoresis.

Gel electrophoresis of the 'type V' fraction, obtained in the above fractionation scheme, indicated the presence of both αA - and αB -chains of type V collagen in a ratio of approx. 1:1.5 (Fig. 1c). The position of elution of these bands was, as expected for type V chains, unaltered by reduction. Similar analysis of the 'type IV' fraction failed to indicate the presence of any component readily recognizable as type IV collagen (Fig. 1d). In previous studies with pig vascular endothelial cells Sankey & Barnes, (1980), we have been able to deduce the synthesis of this collagen by detection in the 'type IV' fraction of a component which after reduction migrated as a species of molecular weight around 140 000. This component appeared not to be present in corneal endothelial cultures.

In a subsequent experiment, cells (representing a new isolation) were labelled either as described above, just before confluence, or after a 7-week period at confluence. After fractionation as described, gel electrophoresis of derived fractions indicated again the synthesis of collagens I, III and V, the relative proportions of which were similar in each case, in this particular experiment being approx. 3:5:2.

Cells freshly isolated were later grown in dishes some of which were precoated with collagen either of interstitial type (type I) or from basement membrane (type IV). In this experiment β -amino-propionitrile (65 μ g/ml) was included in the medium during labelling, and cell layers and media were fractionated separately before analysis by gel electrophoresis. The patterns of synthesis were almost identical irrespective of whether the cells were grown in uncoated dishes or in those pre-treated with either type of collagen. The general pattern was akin to that above. The proportion of collagens, I:III:V, was approx. 1:27:2, suggesting the synthesis by this batch of cells of an even greater relative proportion of type III in comparison with the cells used earlier. Again no type IV was detected. Collagens types I and III were equally distributed between cell layer and medium irrespective of the nature of the substratum to which the cells were attached. However, type V collagen occurred largely (70%) in the cell layer in cultures grown on uncoated dishes, but largely in the medium when dishes were coated

Fig. 1. Analysis of collagen types synthesized by cultured bovine corneal endothelial cells

(a) CM-cellulose chromatography of the fraction precipitated at 0.7 M-NaCl. The position of elution of collagen chains as indicated was established by chromatography of appropriate standards. Fractions under the bar were combined and examined by interrupted electrophoresis (inset), which confirmed the presence in this peak of $\alpha 2(I)$ - and $\gamma(III)$ -chains. The arrows denote in turn the positions of the collagen marker chains $\alpha 1(III)$, $\alpha 1(I)$ and $\alpha 2$. (b) Gel electrophoresis of the 0.7 M-NaCl-precipitated fraction. Direct electrophoresis (continuous line) shows the presence of $\gamma(III)$ and type I chains. The arrows (∇) denote the positions in turn of $\alpha 1(I)$ and $\alpha 2$ marker chains. Interrupted electrophoresis (broken

with collagen (24% only in the cell layer in type I- and 13% in type IV-coated dishes).

Discussion

In contrast with the reported synthesis of type IV collagen by rabbit corneal endothelial cells in culture (Kefalides *et al.*, 1976; Sundar Raj *et al.*, 1979), our studies have revealed the synthesis by the equivalent bovine cells of mainly type III collagen, apparently in the absence of type IV. This result is surprising; the presence of type III collagen has not been reported in Descemet's membrane, which contains predominantly collagen type IV (Kefalides, 1973). In addition to type III collagen, synthesis of some types I and V was detected, compatible with their occurrence in relatively low amounts in Descemet's membrane (Davison & Cannon, 1977). Since completion of these studies, Tseng *et al.* (1981) have reported findings mostly similar to ours. The reason for these contrasting results of the synthesis of either primarily type III or IV collagen is unclear. A similar situation exists in regard to vascular endothelial cells (see the introduction). Our studies do not so far offer any explanation for these differences in terms of either the growth phase of the cells or the nature of the substratum.

Within the same overall pattern of collagen synthesis, we have detected some variation from one batch of cells to the next in the precise amount of one collagen type relative to another. This phenomenon was very marked with pig vascular endothelial cells, where synthesis could vary from largely type I to nearly all type III (Sankey & Barnes, 1980). In this case, type I collagen synthesis has been attributed to the presence of a variant endothelial cell, the so-called 'sprouting cell' (Cotta-Pereira *et al.*, 1980), undergrowing the monolayer. However, with bovine corneal endothelial cells we did not observe 'sprouting', and this phenomenon therefore is unlikely to afford an explanation for type I collagen synthesis by these cells.

In view of these reported differences in the synthetic activity of isolated endothelial cells, culture of these cells should prove to be a useful system in

line) shows the conversion of the γ -component into $\alpha 1(III)$ chains. The arrows (∇) denote in sequence the positions of $\alpha 1(III)$, $\alpha 1(I)$ and $\alpha 2$ markers. (c) Gel electrophoresis of the 'type V' fraction, showing the presence of αA and αB chains of type V collagen. The arrows indicate the positions of αB , αA , $\alpha 1(I)$ and $\alpha 2$ markers respectively. (d) Gel electrophoresis of the 'type IV' fraction after reduction. The arrows indicate the positions of β , $\alpha 1(I)$ and $\alpha 2$ markers.

which to identify factors responsible for the synthesis of one type of collagen in preference to another.

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