Comparison of the collagenous products synthesized in culture by pig aortic endothelial and smooth-muscle cells

Variability in endothelial-cell cultures

Elizabeth A. SANKEY* and Michael J. BARNES†

Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K.

(Received 22 March 1983/Accepted 18 October 1983)

In contrast with smooth-muscle cells from the same tissue, endothelial cells from pig aorta were found to exhibit in culture considerable variability in the pattern of collagen synthesis between one isolation of cells and the next. Synthesis varied from largely collagen type I to virtually all type III in the absence of type I but with small amounts still of collagens types IV and V, to, in one instance, synthesis basically of only type V. Synthesis usually by these cells of collagen predominantly of the interstitial type (I and III) rather than, as might be expected, that from basement membrane (type IV) was not attributable to the influence of subculture. All four collagen types were deposited in the cell layer to an increased extent in primary compared with secondary cultures of either smooth muscle or endothelial origin. Endothelial cells appeared sometimes to synthesize a large- M_r collagenous entity that might conceivably be related to 'short-chain' collagen. In addition, small- M_r hydroxyproline-containing peptides were detected that might reflect rapid collagen(s) turnover in endothelial cultures.

Endothelium is thought to be the source of the underlying basal lamina and, in accord with this, several reports have emphasized the synthesis by cultured endothelium of collagen type IV, the collagenous species characteristic of basement membranes (Howard *et al.*, 1976; Jaffe *et al.*, 1976; Kefalides *et al.*, 1976; Sundar Raj *et al.*, 1979; Madri *et al.*, 1980; Kenney *et al.*, 1981; Sage & Bornstein, 1982). Barnes *et al.* (1978), however, reported that pig aortic endothelial cells synthesized in culture collagens predominantly of the interstitial type, a mixture of types I and III. Subsequent studies have described the synthesis by bovine endothelial cells from aorta, vena cava, capillaries and cornea of largely collagen type III, with some type I in the case of capillary and corneal cells (Sage *et al.*, 1979, 1981a,b; Sankey *et al.*, 1981; Tseng *et al.*, 1981; McAuslan *et al.*, 1982). Relatively small amounts only of collagens types IV and V were produced; in the case of the venous cells and those from capillary (Sage *et al.*,

1981b) and sometimes those of corneal origin (Sankey *et al.*, 1981), synthesis of collagen type IV was not detected at all. The capillary cells also failed to synthesize any collagen type V (Sage *et al.*, 1981b). Knowledge of the factors affecting collagen synthesis by endothelium *in vitro* may be important in understanding control of its synthesis *in vivo*, under conditions of endothelial repair, for example, or in angiogenesis. In the present paper we report that pig aortic endothelial cells, in contrast with smooth-muscle cells from the same tissue, may reveal a marked variation in the pattern of collagen synthesis between one isolation of cells and the next.

A brief account of some of this work has already been given (Sankey & Barnes, 1980).

Materials and methods

Materials

Aortas were obtained from young pigs (approx. 6 months of age) at a local abattoir within 15 min of the animals' death. Dulbecco's Modified Eagle's Medium and foetal-calf serum were obtained from Gibco Europe, Paisley, Strathclyde, Scotland, U.K. Cell-culture ware was from Sterilin, Tedding-

Abbreviation used: SDS, sodium dodecyl sulphate.

* Present address: The London Hospital, London E1 1BB, U.K.

† To whom reprint requests should be sent.

ton, Middx., U.K. Collagenase (type IV) for endothelial-cell isolation was from Sigma Chemical Co., Poole, Dorset, U.K., and pepsin was from Millipore, Harrow, Middx., U.K. L-[G-³H]Proline was purchased from Amersham International, Amersham, Bucks., U.K.

Isolation of cells and culture conditions

Aortic smooth-muscle cells were obtained, as in previous work (Barnes *et al.*, 1976), from medial explants prepared from pig aortas basically as described by others (Jarmolych *et al.*, 1968; Ross, 1971). After subculture, cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) foetal-calf serum and 2 mM-L-glutamine (Eagle *et al.*, 1956). To investigate collagen synthesis, cells (generally in third passage) were grown (in 25 cm² culture flasks, six to ten per experiment) for 48 h, just before confluence, in media (4 ml per flask) containing L-[G-³H]proline (generally at 15–40 µCi/ml) and supplemented with penicillin-G (100 units/ml), streptomycin sulphate (100 µg/ml), ascorbic acid (50 µg/ml) and glutamine [40 µM; see Ronnema *et al.* (1977) and Sankey (1981)]. Further supplements of ascorbic acid and glutamine (at the concentrations specified) were made at 12 h intervals during the labelling period.

Aortic endothelial cells were obtained, as previously (Barnes *et al.*, 1978), from pig aortas by a procedure, basically that introduced by Gimbrone *et al.* (1974) and Jaffe *et al.* (1973), involving a brief (7 min) digestion of the luminal surface of the vessel wall at 37°C with collagenase (0.1%, w/v). After subculture, cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) foetal-calf serum and 40 µM-glutamine. To investigate collagen synthesis, cultures were labelled with L-[G-³H]proline as described above for smooth-muscle cells.

Analysis of radiolabelled collagens

All procedures were carried out at 4°C unless otherwise stated. Cell layers and media were generally processed separately. At the completion of radiolabelling of cultures, cell layers were collected into 0.5 M-acetic acid and dispersed with a glass/Teflon homogenizer. Cell layers and media were dialysed extensively first against 0.15 M-acetic acid containing L-proline (0.5 mg/ml) and then against 0.5 M-acetic acid. Samples were digested with pepsin (100 µg/ml) as described previously (Barnes *et al.*, 1976; Sankey *et al.*, 1981), cell-layer samples (in the presence of carrier collagen at 1 mg/ml) for 16 h at 4°C, and samples of medium for 6 h at 9°C. After pepsin digestion, samples were then adjusted to pH 8.0 and left for 20 min to inactivate the enzyme. After dialysis of samples

against sodium phosphate buffer, pH 7.6 and 10.4, radiolabelled collagens were precipitated overnight in the presence of unlabelled carrier collagen from pig aorta (Sankey *et al.*, 1981) by addition of NaCl to 30% (w/v). The precipitate was redissolved in phosphate buffer, and collagens were precipitated a second time by a modification of the gelation procedure of Trelstad & Lawley (1977), involving, as detailed previously (Barnes *et al.*, 1978), dilution of the solution to 1.0, incubation at 37°C for 6 h and then dialysis overnight at room temperature against 10 vol. of distilled water.

In some instances, precipitated collagens were further fractionated as previously described (Sankey *et al.*, 1981), by a procedure, basically that of Kresina & Miller (1979), designed to separate interstitial collagens from other collagen types. Briefly, samples dissolved in 0.5 M-acetic acid were precipitated successively firstly at 0.7 M-NaCl (to precipitate collagen types I and III) and then 1.2 M-NaCl (to precipitate collagen types IV and V). Collagen type V was then separated from type IV by dialysis of the latter fraction against 0.02 M-NaCl/2 M-urea/0.01 M-Tris/HCl, pH 8.6, to precipitate collagen type V (Kresina & Miller, 1979).

Fractions arising from these procedures were examined by gel filtration, CM-cellulose ion-exchange chromatography and/or SDS/polyacrylamide-gel electrophoresis as described below.

Procollagen analysis

In some instances samples of the medium after radiolabelling were analysed without prior pepsin treatment for the presence of procollagens (Burke *et al.*, 1977). In these cases proteinase inhibitors, namely EDTA (50 mM), phenylmethanesulphonyl fluoride (1 mM) and *N*-ethylmaleimide (50 µg/ml), were added to the medium at the end of the labelling period. Samples were precipitated with (NH₄)₂SO₄ (20%, w/v), and each precipitate was redissolved in phosphate buffer, pH 7.6 and 10.4 (containing inhibitors), before DEAE-cellulose chromatography as described below.

Gel filtration

Samples after denaturation were examined by gel filtration as previously described (Barnes *et al.*, 1976, 1978), on a column (120 cm × 1.5 cm) of Sepharose CL-6B eluted with 0.1 M-sodium phosphate buffer, pH 7.4, containing 0.1% SDS.

CM-cellulose chromatography

This was undertaken under denaturing conditions precisely as described earlier (Barnes *et al.*, 1976, 1978).

SDS/polyacrylamide-gel electrophoresis

This was performed in a 6% acrylamide gel as detailed previously (Sankey *et al.*, 1981; Morton &

Barnes, 1982). In some cases electrophoresis was interrupted to permit reduction *in situ* with 2-mercaptoethanol as described by Sykes *et al.* (1976). The sectioning of gels and the measurement of the radioactivity in individual gel slices were as described previously (Sankey *et al.*, 1981).

DEAE-cellulose chromatography

This was undertaken at 4°C as described by Burke *et al.* (1977) on a column (1 cm × 10 cm) of DEAE-cellulose eluted with a linear gradient of 0–0.2M-NaCl in 0.05M-Tris/HCl buffer, pH 7.5, containing 4M-urea, over a total volume of 400 ml. Before application samples were dialysed against starting buffer [containing proteinase inhibitors (Burke *et al.*, 1977)]. Fractions of volume 5 ml were collected. Radioactivity in the eluate was measured as in other procedures by counting that in a 0.5 ml sample of each 5 ml fraction in 5 ml of scintillation fluid [3 vol. of Pico-Fluor 30 (Packard Instrument Co., La Grange, IL, U.S.A.) diluted with 2 vol. of scintillation-grade toluene] in a Rack-Beta mini-vial liquid-scintillation counter (LKB Instruments, Croydon, Surrey, U.K.).

Determination of proline and hydroxyproline radioactivities

Conditions of hydrolysis and the measurement of proline and hydroxyproline radioactivities in hydrolysates were as previously described (Barnes *et al.*, 1978).

Collagenase digestion

Purification of collagenase (CLSPA from Millipore, Harrow, Middx., U.K.) to remove non-specific proteolytic activity and conditions of digestion were as previously described (Barnes *et al.*, 1978).

Results

Collagen synthesis by smooth-muscle cells

Analysis of smooth-muscle-cell cultures by precipitation of radiolabelled collagens (as described in the Materials and methods section) and their examination by gel filtration and CM-cellulose chromatography indicated, as in a previous study based on these procedures (Barnes *et al.*, 1976), the synthesis predominantly of collagens types I and III in a ratio of approx. 3:1. Subsequent fractionation by the procedure of Kresina & Miller (1979) and analysis by SDS/polyacrylamide-gel electrophoresis confirmed this result and also permitted the detection of the synthesis of relatively small amounts of collagens types IV and V (Fig. 1). The $\alpha 1(I)/\alpha 2$ radioactivity ratio (measured as 3.5:1, for example, in the experiment results for which are given in Fig. 1) indicated the synthesis, in addition to collagen type I, of some

type I trimer, $[\alpha 1(I)]_3$, a finding previously reported for monkey smooth-muscle cells (Mayne *et al.*, 1978). The $\alpha 1(V)/\alpha 2(V)$ radioactivity ratio, generally close to 2:1, was indicative of the synthesis of collagen type V of chain composition $[\alpha 1(V)]_2\alpha 2(V)$. Synthesis of type V collagen by smooth-muscle cells has been reported previously for cells from monkey aorta (Mayne *et al.*, 1978). Type IV collagen was identified tentatively as a collagenase-sensitive polypeptide, of M_r 140 000 after reduction of disulphide bonds, occurring in the 'type IV' fraction. A similar entity identified in endothelial cultures has been attributed to type IV collagen synthesis (Sage *et al.*, 1979, 1981a,b). On the basis of the radioactivity measured in individual collagen chains after their separation by SDS/polyacrylamide-gel electrophoresis, it was calculated that collagens were synthesized in the following very approximate proportions, each expressed as a percentage of the total: collagen type I, 70; type III, 25; type V, 5; type IV, less than 1. This overall pattern of synthesis was maintained from one isolation of cells to the next. All four collagens occurred predominantly in the medium (Table 1).

Cells in primary culture exhibited the same pattern of synthesis, but an increased proportion of each collagen was deposited in the cell layer (Table 1), with the overall result that, for instance in the example documented in Table 1, 62% of the total collagen occurred in the cell layer as opposed to 13% in secondary culture.

Collagen synthesis by endothelial cells

On the basis of proline and hydroxyproline radioactivities, collagen in endothelial cultures represented only 4–5% of total protein synthesis as opposed to about 25% in smooth-muscle-cell cultures. Considerable variation was observed in the pattern of collagen synthesis between one isolation of endothelial cells and the next. In all, results for four consecutive isolations are given below.

Analysis of batch 1 secondary cell cultures initially by precipitation of radiolabelled collagens (as described above) and their examination by CM-cellulose chromatography and gel filtration indicated, as in an earlier study based on these procedures (Barnes *et al.*, 1978), synthesis predominantly of collagen type I. Subsequent fractionation by the procedure of Kresina & Miller (1979) and analysis then by SDS/polyacrylamide-gel electrophoresis indicated the synthesis of collagens types I, III, IV and V in the following very approximate percentages relative to the total: type I, 74; type III, 12; type IV, 2; type V, 12. Separation of these four collagens by SDS/polyacrylamide electrophoresis after their fractionation was exactly as illustrated in Fig. 1 for smooth-

Fig. 1. SDS/polyacrylamide-gel electrophoresis of the radiolabelled collagenous products synthesized by pig aortic smooth-muscle cells in secondary culture

The culture medium, after pepsin digestion, was fractionated as described in the Materials and methods section to yield the following. (a) An interstitial-collagen fraction precipitated from acid pH (0.5M-acetic acid) at 0.7M-NaCl; collagen type III α -chains were separated from the $\alpha 1$ - and $\alpha 2$ -chains of type I, as illustrated, by converting the γ -chains of collagen type III into α -size by reduction with mercaptoethanol after commencement of electrophoresis (interrupted electrophoresis). (b) A collagen type V fraction precipitated at 1.2M-NaCl and insoluble in 0.02M-NaCl/2M-urea in 0.01M-Tris/HCl, pH 8.6; $\alpha 1(\text{V})$ - and $\alpha 2(\text{V})$ -chains were identified. (c) A type IV fraction precipitated at 1.2M-NaCl but soluble in 0.02M-NaCl/2M-urea in 0.01M-Tris/HCl, pH 8.6; type IV was identified as a peak of M_r 140000 (arrowed) appearing after reduction. —, Profile obtained in the absence of reduction; ----, profile obtained after reduction (before electrophoresis). Cell layers were similarly analysed. The positions of collagen marker chains, indicated by arrows, were established by electrophoresis of appropriate collagen standards, either contained within the sample itself as carrier protein(s) or run alongside in an adjacent lane. Standards were detected by staining with Coomassie Blue.

Table 1. Analysis of collagens synthesized by pig aortic smooth-muscle and endothelial cells in culture

		Collagen type . . .				Total (I + III + IV + V)	
Smooth-muscle cells		I	III	IV	V		
Primary culture:							
% of total		65	30	0.4	5		
% in medium		39	30	89	58		38
Secondary culture:							
% of total		69	27	0.2	4		
% in medium		87	85	100	90		87
Endothelial cells							
Primary culture (batch 2 cells):							
% of total		72	21	5	2		
% in medium:		43	50	90	57		47
Secondary culture (batch 1 cells):							
% of total		74	12	2	12		
% in medium		70	64	95	73		70

muscle-cell cultures thought to reflect type IV synthesis.

Batch 2 cells, examined in this case in primary culture, gave results almost identical with those for batch 1 cells except that, as noted for primary smooth-muscle cells, deposition of collagens in the cell layer was increased (Table 1).

Batch 3 cells, in contrast, synthesized predominantly collagen type III to the exclusion of type I. This was indicated by CM-cellulose chromatography and gel filtration (Fig. 2), by procollagen analysis on DEAE-cellulose (Fig. 3) and by analysis by SDS/polyacrylamide-gel electrophoresis (after fractionation as described by Kresina & Miller, 1979), which indicated synthesis of collagens type III, IV and V in the proportions of one to the other of very approximately 90:1:9 (Fig. 4).

Batch 4 cells, on the other hand, appeared to synthesize little if any interstitial collagen and no type IV collagen. Thus gel filtration indicated the presence of relatively little material of γ - or α -chain size, and SDS/polyacrylamide-gel electrophoresis after fractionation as before permitted the detection of neither collagens types I and III in the 0.7M-NaCl-precipitated fraction nor the 140000- M_r species in the 'type IV' fraction, and indicated synthesis basically of only collagen type V.

Fig. 2. CM-cellulose chromatography of the radiolabelled collagenous products synthesized by pig aortic endothelial (batch 3) cells in culture

Collagenous material in the culture medium, after pepsin digestion, was twice precipitated, as described in the Materials and methods section, and the precipitated material was then subjected to chromatography under denaturing conditions as described in the text. The positions of elution of collagen chains as indicated were established by chromatography of appropriate collagen standards. The identification of the major radioactive peak as collagen type III was confirmed by its examination by gel filtration, which showed it to be largely of γ -chain size, which could be decreased to α -size by reduction of disulphide bonds with mercaptoethanol.

Examination of the culture medium on DEAE-cellulose (as described in the Materials and methods section) indicated that, in contrast with batch 3 cultures (see Fig. 4), a high proportion (80%) of the total radioactivity was unretained. Examination of this material by SDS/polyacrylamide-gel electrophoresis indicated the presence of a large- M_r component that after either reduction with mercaptoethanol or mild treatment with pepsin (100 μ g/ml; 6 h at 4°C) exhibited a mobility intermediate between those of collagen γ - and β -chains (Fig. 5). This component was found to be collagenase-sensitive. A combination of pepsin treatment and reduction yielded relatively small peptides (of undetermined size), as shown in Fig. 5.

Other hydroxyproline-containing components

Although precipitation by gelation was originally undertaken in the hope of separating the interstitial collagens from collagen types IV and V (Trelstad & Lawley, 1977), it became apparent that the procedure as employed (Barnes *et al.*, 1978) precipitated all four collagens. Material unprecipitated was of small M_r (predominantly 55000 and 20000 in size by gel filtration) and contained relatively little radioactive hydroxyproline. Material precipitated by gelation from the medium of smooth-muscle-cell cultures exhibited a proline/hydroxyproline radioactivity ratio close to unity. The equivalent ratio in endothelial cultures, however, was higher (2:1, for example, in batch 1 cultures), indicative of the presence of some non-collagenous material or of collagenous species of low hydroxyproline content. Gel filtration revealed the presence, in addition to γ - and α -sized chains, of components 55000 and 24000 in size, especially evident in batch 2 and batch 4 cultures (Fig. 6). The proline/hydroxyproline radioactivity ratios in these (measured in batch 1 cultures) were 3:1 and 12:1 respectively. Collagenase digestion in each case released most of the hydroxyproline radioactivity in diffusible form, leaving a collagenase-resistant entity of unaltered size, indicating the presence (before digestion) of a mixture of collagen-derived and non-collagen-derived peptides of similar size. A component of 24000 was also noted in the cell-layer samples of both smooth-muscle-cell and endothelial-cell cultures, but the proline/hydroxyproline radioactivity ratio was relatively high (50:1 or more).

Discussion

Reason(s) for the variation in collagen synthesis in endothelial cultures is (are) not known. Differences in the proportion of collagen type I relative to other collagens could conceivably be due to the

Fig. 3. SDS/polyacrylamide-gel electrophoresis of the radiolabelled collagenous products synthesized by pig aortic endothelial (batch 3) cells in culture

Culture medium and cell-layer samples were processed separately by employing the fractionation procedure detailed in the legend to Fig. 1. (a) The interstitial-collagen fraction derived from the medium (—) and cell layer (----) examined by electrophoresis interrupted to permit reduction *in situ*. (b) The type V fraction derived from the medium (—) and cell layer (----). (c) The type IV fraction derived from the medium and examined in the absence of reduction (—) or after reduction (before electrophoresis) with mercaptoethanol (----). (d) The type IV fraction from the cell layer, reduced (----) and unreduced (—). Within each fraction, medium and cell-layer samples analysed

presence of endothelial 'sprouting' cells, which have been reported to synthesize this collagen in preference to type III collagen produced by the 'normal' cell (Cotta-Pereira *et al.*, 1980; McAuslan *et al.*, 1982). We have noticed the presence of 'sprouting' cells in our post-confluent cultures (Sankey, 1981). However, pre-confluent cultures, whether synthesizing predominantly collagen type I or primarily collagen type III to the exclusion of type I, were both equally prone to 'sprouting' after confluence. Furthermore, we have not seen 'sprouting' cells in corneal endothelial-cell cultures (Sankey, 1981), despite the synthesis by such cultures of some collagen type I (Sankey *et al.*, 1981).

We do not know whether synthesis predominantly of interstitial collagens (type I and/or type III) rather than collagen type IV results from an

Fig. 4. DEAE-cellulose chromatography of the radiolabelled collagenous products synthesized by pig aortic endothelial (batch 3) cells

Collagenous material in the culture medium, collected in the presence of proteinase inhibitors, was precipitated (without prior pepsin digestion), and the precipitated material was subjected to chromatography as described in the Materials and methods section to permit the separation of procollagens. About one-tenth of the radioactivity applied was unretained on the column. This was shown by gel filtration to be of small M_r (15000–20000). After the collection of the unretained material, a salt gradient was then introduced, which resulted in the elution of a single major peak, as illustrated, occurring in the position expected for procollagen type III.

represented an equal proportion of the respective total. The positions of collagen marker chains are indicated. The presence of a small peak in the interstitial-collagen fraction in the position of $\alpha 1(I)$ -chains, despite the absence of type I collagen, as indicated by CM-cellulose chromatography (Fig. 2), may suggest the presence of some type III α -chains before reduction.

Fig. 5. SDS/polyacrylamide-gel electrophoresis of the radiolabelled material synthesized in culture by pig aortic endothelial (batch 4) cells and unretained on DEAE-cellulose

Collagenous material in the culture medium, collected in the presence of proteinase inhibitors, was precipitated, and the precipitated material was subjected to chromatography as described in the Materials and methods section. The unretained radioactivity was collected and the material examined by SDS/polyacrylamide-gel electrophoresis. (a) Sample examined in the absence of reduction (—) and after reduction (before electrophoresis) with mercaptoethanol (----). (b) Sample subjected to mild pepsin treatment and then examined in the absence of reduction (—) or after reduction (before electrophoresis) with mercaptoethanol (----).

alteration in phenotype as a consequence of culture. If this is so, then the alteration must occur relatively rapidly, since this pattern of synthesis was observed in primary culture as well as in secondary culture. In view of the presence (as reported by Gay *et al.*, 1975) of type III collagen (without type I) in the subendothelium of the young vessel wall (where little if any intimal thickening would be expected and significant migration of smooth-muscle cells into the intimal space seems unlikely), it is possible that this collagen type represents a major collagenous product at least of aortic endothelial cells *in vivo*. This does not, of course, exclude the possibility that the endothelium is also responsible for the synthesis of basement-membrane components, including collagen type IV.

It may be argued that the presence of relatively small amounts of collagen type IV could be attributed to its pepsin-sensitivity. However, the

Fig. 6. Gel filtration of the radiolabelled collagenous products synthesized by pig aortic endothelial (batch 2) cells in culture

Collagenous material in the culture medium, after pepsin digestion, was twice precipitated, as described in the Materials and methods section, and the precipitated material was subjected to gel filtration under denaturing conditions as described in the text. The results illustrate the presence in predominant amounts of components of M_r 55000 and 24000.

conditions of pepsin digestion employed in the present work were comparatively mild and would not be expected to result in extensive fragmentation of the type IV molecule (Kuhn *et al.*, 1981; Bentz *et al.*, 1983). We therefore consider that the 140000- M_r moiety may represent a reasonable estimate of total collagen type IV synthesis. Substantial losses of type III collagen as a result of pepsin degradation also seem unlikely (Burke *et al.*, 1977; Mayne *et al.*, 1978).

The reason for the lack of interstitial-collagen synthesis sometimes, as in batch 4 cells, is also unclear. Variations such as these may conceivably arise from the selection of differing subpopulations of endothelial cells during culture. Such subpopulations may perhaps reflect the derivation of cells from different regions of the aorta, although it should be emphasized that the same overall region of the vessel was used for each cell isolation. Alternatively, variations between cultures may reflect changes in the degree of intracellular degradation of one collagen relative to another.

Variations such as those described in the present paper for pig aortic endothelial cells may occur in other species, since the equivalent bovine cell has been variously reported by different laboratories to synthesize predominantly collagen type III, type IV or type V or type I in the case of 'sprouting' (Howard *et al.*, 1976; Sage *et al.*, 1979, 1981a,b; Cotta-Pereira *et al.*, 1980; Madri *et al.*, 1980; McAuslan *et al.*, 1982).

The collagenous material unretained on DEAE-cellulose (especially evident in batch 4 cells) may

reflect the synthesis of 'short-chain' or 'intimal' collagen, a large- M_r species identified in pepsin digests of intima or placenta containing disulphide bonds, reduction of which yields peptides of M_r values 40000–55000 (Chung *et al.*, 1976; Furuto & Miller, 1980; Jander *et al.*, 1981). It seems unlikely to be endothelial collagen (M_r 177000), which is non-reducible and extremely sensitive to pepsin (Sage *et al.*, 1980), nor collagen type IV, in view of the absence of material of around α -chain size or greater after combined treatment with pepsin and mercaptoethanol.

The origin of the small- M_r hydroxyproline-containing peptides present sometimes in relatively large amounts in collagen precipitates from endothelial culture is unknown, but presumably they may reflect relatively high turnover of collagen(s) in endothelial cultures.

The occurrence of collagens type IV and V predominantly in the medium is in contrast with reports by others (Sage *et al.*, 1979, 1981a,b; Madri *et al.*, 1980) of their total deposition in the cell layer of endothelial cultures. Factors controlling the deposition of collagens in culture may presumably include the rate of processing of precursor procollagen molecules, the rate of fibrillogenesis, the degree of intermolecular cross-linking and the extent to which uncleaved procollagen molecules may be rendered insoluble by intermolecular disulphide bonds. Which of these factors, if any, is responsible for the increased deposition that we noted, often quite marked, of collagens into the cell layer of primary cultures is at present unknown.

The support of the British Heart Foundation is gratefully acknowledged. M. J. B. is a member of the External Scientific Staff of the Medical Research Council.

References

- Barnes, M. J., Morton, L. F. & Levene, C. I. (1976) *Biochem. Biophys. Res. Commun.* **70**, 339–347
- Barnes, M. J., Morton, K. F. & Levene, C. I. (1978) *Biochem. Biophys. Res. Commun.* **84**, 646–653
- Bentz, H., Morris, N. P., Murray, L. W., Sakai, L. Y., Hollister, D. W. & Burgeson, R. E. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3168–3172
- Burke, J. M., Balian, G., Ross, R. & Bornstein, P. (1977) *Biochemistry* **16**, 3243–3249
- Chung, E., Rhodes, R. K. & Miller, E. J. (1976) *Biochem. Biophys. Res. Commun.* **71**, 1167–1174
- Cotta-Pereira, G., Sage, H., Bornstein, P., Ross, R. & Schwartz, S. (1980) *J. Cell. Physiol.* **102**, 183–191
- Eagle, H., Oyama, V. I., Levy, M., Horton, C. L. & Fleischman, R. J. (1956) *J. Biol. Chem.* **218**, 607–616
- Furuto, D. K. & Miller, E. J. (1980) *J. Biol. Chem.* **255**, 290–295
- Gay, S., Balleisen, L., Remberger, K., Fietzek, P. P., Adelman, B. C. & Kuhn, K. (1975) *Klin. Wochenschr.* **53**, 899–902
- Gimbrone, M. A., Cotram, R. S. & Folkman, J. (1974) *J. Cell Biol.* **60**, 673–684
- Howard, B. V., Macarak, E. J., Gunson, D. & Kefalides, N. A. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2361–2364
- Jaffe, E. A., Nachman, R. L., Becker, C. G. & Minick, C. R. (1973) *J. Clin. Invest.* **52**, 2745–2756
- Jaffe, E. A., Minick, C. R., Adelman, B. C., Becker, C. G. & Nachman, R. (1976) *J. Exp. Med.* **144**, 209–225
- Jander, R., Rauterberg, J., Voss, B. & von Bassewitz, D. B. (1981) *Eur. J. Biochem.* **114**, 17–25
- Jarmolych, J., Daoud, A. S., Landau, J., Fritz, K. E. & McElvene, E. (1968) *Exp. Mol. Pathol.* **9**, 171–188
- Kefalides, N. A., Cameron, J. D., Tomich, E. A. & Yanoff, M. (1976) *J. Biol. Chem.* **251**, 730–733
- Kenney, M. C., Benya, P. D., Nimni, M. E. & Smith, R. E. (1981) *Exp. Eye Res.* **33**, 131–140
- Kresina, T. F. & Miller, E. J. (1979) *Biochemistry* **18**, 3089–3097
- Kuhn, K., Wiedemann, H., Timpl, R., Risteli, J., Dieringer, H., Voss, T. & Glanville, R. W. (1981) *FEBS Lett.* **125**, 123–128
- Madri, J. A., Dreyer, B., Pitlick, F. A. & Furthmayr, H. (1980) *Lab. Invest.* **43**, 303–315
- Mayne, R., Vail, M. S. & Miller, E. J. (1978) *Biochemistry* **17**, 446–452
- McAuslan, B. R., Hannan, G. N. & Reilly, W. J. (1982) *J. Cell. Physiol.* **112**, 96–106
- Morton, L. F. & Barnes, M. J. (1982) *Atherosclerosis* **42**, 41–51
- Ronnemaa, T., Vihersaari, T. & Ranta, H. (1977) *Scand. J. Clin. Lab. Invest.* **37**, 351–356
- Ross, R. (1971) *J. Cell Biol.* **50**, 172–186
- Sage, H. & Bornstein, P. (1982) *Arteriosclerosis* **2**, 27–36
- Sage, H., Crouch, E. & Bornstein, P. (1979) *Biochemistry* **18**, 5433–5442
- Sage, H., Pritzl, P. & Bornstein, P. (1980) *Biochemistry* **19**, 5747–5755
- Sage, H., Pritzl, P. & Bornstein, P. (1981a) *Biochemistry* **20**, 436–442
- Sage, H., Pritzl, P. & Bornstein, P. (1981b) *Arteriosclerosis* **1**, 96–106
- Sankey, E. A. (1981) Ph.D. Thesis, University of Cambridge
- Sankey, E. A. & Barnes, M. J. (1980) *Proc. Eur. Symp. Connect. Tissue Res.* **7th**, 52–53
- Sankey, E. A., Bown, F. E., Morton, L. F., Scott, D. M. & Barnes, M. J. (1981) *Biochem. J.* **198**, 707–710
- Sundar Raj, C. V., Freeman, I. L., Church, R. L. & Brown, S. I. (1979) *Invest. Ophthalmol. Visual Sci.* **18**, 75–84
- Sykes, B., Puddle, B., Francis, M. & Smith, R. (1976) *Biochem. Biophys. Res. Commun.* **72**, 1472–1480
- Trelstad, R. L. & Lawley, K. R. (1977) *Biochem. Biophys. Res. Commun.* **76**, 376–384
- Tseng, S. C. G., Savion, N., Gospodarowicz, D. & Stern, R. (1981) *J. Biol. Chem.* **256**, 3361–3365