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SYNTHESIS OF INTERSTITIAL COLLAGENS BY PIG AORTIC ENDOTHELIAL CELLS IN CULTURE

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SUMMARY: Examination of the collagens synthesized by pig aortic endothelial cells in culture and precipitated from either the cell layer or medium, following pepsin digestion, demonstrated that the major species was Type I together with some Type III collagen and α1 (I) trimer. Additional chains in the cell layer chromatographing with Type I α1-chains on CM cellulose may be partly derived from basement-membrane-associated collagen but in the medium would appear to be entirely derived from α1 (I) trimer. These results imply that the endothelial cell may secrete the Type III collagen located in the immediate subendothelial space and, in part at least, the Types I and III occurring in diffusely thickened intima and the atherosclerotic plaque.

Endothelial cells in culture synthesize collagen (1,2,3) that has been described as of basement-membrane-type (1,2) in accord with the derivation of the vascular basement membrane from the endothelium. This communication offers the first definitive evidence that cultured endothelial cells can synthesize, additionally, the interstitial collagens (Types I and III). Our findings permit the inference that this cell type may secrete the Type III collagen that has been located immunohistochemically in the immediate sub-endothelial space in the blood vessel wall (4) and (or at least in part) the Types I and III collagens that have been detected by chemical analysis in diffusely thickened human intima (authors' unpublished data) and in the atherosclerotic plaque (5). Collagen at these sites has been considered to originate primarily from the medial smooth muscle cell (6).

MATERIALS AND METHODS: Culture of endothelial cells Endothelial cells were obtained as before (3) from pig aorta by the method of Jaffe et al (7) as modified by Leake and Bowyer (8). Endothelial cells so isolated and then subcultured 5-6 times in 199 medium containing 20% foetal calf serum (FCS), were kindly provided by the latter authors. For the purposes of the present investigation, cells were grown in 60 mm Falcon dishes at an initial density of 50,000 in 4 ml of Dulbecco and Vogt's modification of Eagle's MEM containing 10% FCS. Medium was exchanged for fresh every three days. After six days, ascorbic acid (50 μg/ml) was added to the medium. At specified days, indicated later, medium was replaced with fresh containing either L- [G-^{3}H] proline or L- [4,5-^{3}H]lysine (generally 0.5 mCi/dish) and ascorbic acid (50μg/ml). After 24h, plates were either

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harvested or a further supplement of ascorbic acid was given. In the latter case dishes were harvested 24h later. Radiolabelled collagens in the cell layer and medium were examined as described below.

Examination of cell layer collagens Digestion of cell layers, dispersed in 0.5M acetic acid, with pepsin, precipitation of the digest at 0.9M NaCl and the separation of precipitated collagens by differential salt precipitation, all in the presence of carrier were undertaken as before (9). Fractions precipitating at 1, 1.71, 2.1 and 2.56M NaCl were collected and examined by CM cellulose chromatography.

Examination of collagens in the medium The medium following dialysis was digested with pepsin as described previously (9). The digest was dialysed against 0.4I Na phosphate buffer, pH 7.6. After addition of carrier collagen (prepared by precipitation of a pepsin digest of pig aorta by dialysis against 0.02M Na_2HPO_4) at approx 3mg/ml, collagens in the digest were precipitated by dialysis against 0.02M phosphate. The precipitate was redissolved in the 0.4I buffer and then incubated overnight at 37° (10) to effect the thermal gelation of interstitial collagens. The gel was centrifuged for 30 min at 10,000 g at 25°. The residue (containing interstitial collagens) and supernatant were examined by CM cellulose chromatography.

A portion of the supernatant was subjected to a second gelation by means of a modification of the above procedure (Dr. J. Rauterberg, unpublished data). The sample after addition of carrier collagen at approx 3 mg/ml was diluted to 0.2I , incubated at 37° for 6h and then dialyzed at 20° against 10 vol H_2O overnight. The precipitate was collected by centrifugation as above.

CM cellulose chromatography was exactly as before (9). Radioactivity in the eluate was measured as before (11) except that PCS (Amersham/Searle Corp.) diluted 2:1 (v/v) with xylene, was used as scintillant.

Gel chromatography was undertaken with a column (140 x 1.5cm) of Sepharose CL-6B; conditions were otherwise as before (9). Radioactivity in the eluate was measured as above.

Collagenase digestion Samples of the cell layer, after dispersal (10), or medium or fractions derived therefrom were dialysed against 0.05M tris-HCl, pH7.6 (at 25°) containing 0.01M Ca acetate and 0.02% Na azide, denatured at 100° for 5 min, and then redialyzed. Collagenase (Worthington's CLSPA purified and treated as described by Benya et al (12))was added at 20 units/ml. Samples were incubated for 6h at 37°, then dialyzed against water and the diffusate in each case lyophilized prior to hydrolysis.

Hydrolysis Samples were heated in 6N HCl in sealed tubes at 108° for 24h. Hydrolysates were dried in vacuo in a dessicator over conc. H_2SO_4 in the presence of NaOH pellets.

Measurement of lysine and hydroxylysine radioactivity in hydrolysates was as before (11).

Measurement of proline and hydroxyproline radioactivity in hydrolysates. Proline and hydroxyproline were separated on a Technicon Amino Acid Autoanalyzer at 60° using the standard citrate buffer, pH 2.9. Radioactivity in the eluate was counted as above.

RESULTS: In initial studies the % conversion of lysine to hydroxylysine in collagenase-susceptible material in cultures labelled with [^{3}H] lysine during the period indicated was as follows:-

Expt. 1; 5-7d: Cell layer, 19; Medium, 24. 10-12d: Cell layer, 26; Medium, 23.

Expt. 2; 6-7d: Cell layer, 5; Medium, 44. 12-13d: Cell layer, 15; Medium, 52.

Since basement-membrane (Type IV) collagen exhibits values of about 80% (13-17)

the figures above were taken to indicate the synthesis of interstitial collagens. This was confirmed by a more detailed analysis of the collagens synthesized in [^{3}H] proline-labelled cultures as described below.

Examination of radio-labelled collagens in the cell layer In two separate experiments, approx. two-thirds of the total [^{3}H] hydroxyproline in the pepsin digest was in material that precipitated at 0.9M NaCl. Of the remainder, there was negligible precipitation upon dialysis against 0.02M $Na_2 HPO_4$ (18). The pro/hyp radioactivity ratio (of approx 2:1 and 4:1 respectively) obtained following collagenase treatment suggested the unprecipitated collagenous moiety was under-hydroxylated material and, certainly,was not obviously basement-membrane-related (Type IV) collagen (where a ratio appreciably less than one might be anticipated (13-17)). This material was examined no further.

In Expt. 1 (labelled from 10-12d), the % distribution of total radio-activity following salt fractionation of collagens precipitated at 0.9M NaCl was as follows:-

1M ppt, 4; 1.71M ppt, 16; 2.1 M ppt, 25; 2.56 M ppt, 34; 2.56M - soluble, 21.

In accord with known precipitation behaviour, chromatography on CM cellulose (Fig. 1) revealed the presence of Type III collagen in the 1.71M fraction and α1 (I) and α2 chains from Type I in the 2.56M fraction. The α1:α2: ratio of 5 in the latter fraction indicated an excess of chains in the α1(I) region, suggesting the presence either of Type I (a1) trimer (which usually precipitates at a higher molarity than 2.56M NaCl (19-23) but can in part coprecipitate with normal Type I (24)) or perhaps Type IV collagen which is known to precipitate generally around 2M NaCl (25-27) and α-chains from which have been shown to chromatograph in the vicinity of α1 (I) chains (13).

In Expt. 2 (labelled from 9-11d), the % distribution of radioactivity was as follows:-

1M ppt, 9; 1.71 M ppt, 17; 2.1 M ppt, 54; 2.56 M ppt, 4; 2.56 M-soluble, 16.

In this instance , most of the radioactivity precipitated at 2.1M NaCl. CM cellulose chromatography again indicated the presence of Type III collagen

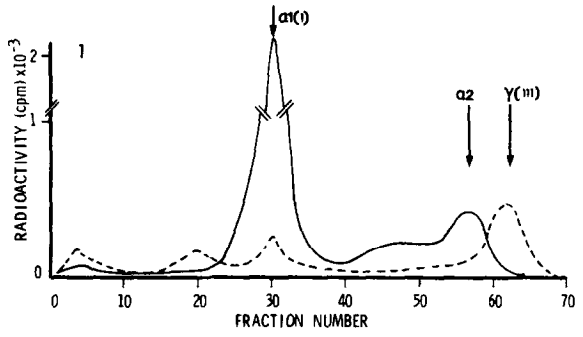

Figure 1. Endothelial cells in culture: CM cellulose chromatography of the precipitates at 1.71 and 2.56M NaCl obtained in the fractionation of radiolabelled cell layer collagens isolated after pepsin treatment (Expt. 1). Radioactivity in a 0.5 ml aliquot of each 10 ml fraction of the eluate was counted in 10 ml of scintillant at 30% efficiency. The position of marker chains as indicated was established by chromatography of appropriate unlabelled collagens. The results relate to the cell layers pooled from three plates, each labelled with 0.3 mCi[L G-^{3}H] proline from 10-12d.

---------- 1.71M ppt. _____ 2.56M ppt.

in the 1.71M fraction (not shown). In the 2.1M fraction, radioactivity was located in α1(I) and α2 regions indicative again of Type I collagen (Fig. 2). The elevated α1:α2 ratio (approx. 5) was again noted. The α2-peak was essentially of α-chain size by gel filtration. However, the α1-peak revealed a complex pattern with peaks of α-chain size and greater (Fig. 2), perhaps suggesting excess chains in the regionof α1(I) were attributable to Type IV collagen rather than Type I trimer (14-17,28). However the pro/hyp radioactivity ratio was close to unity in all of these peaks. The 2.56M fraction revealed only a single peak on CM cellulose in the position of α1 (I)-chains (not shown) and since this represents material that precipitated after Type I, is considered to be α1(I)-trimer.

The nature of the collagenous material soluble at 2.56M NaCl is uncertain. The pro/hyp radioactivity ratio of 1.4 was compatible with its identity as α1 (I) trimer. However CM cellulose chromatography exhibited only a single peak at the front and failed to reveal either α1 (I) chains or α[A] and α[B] chains derived from Type V collagen which could also in theory occur in this fraction (18,24,29,30).

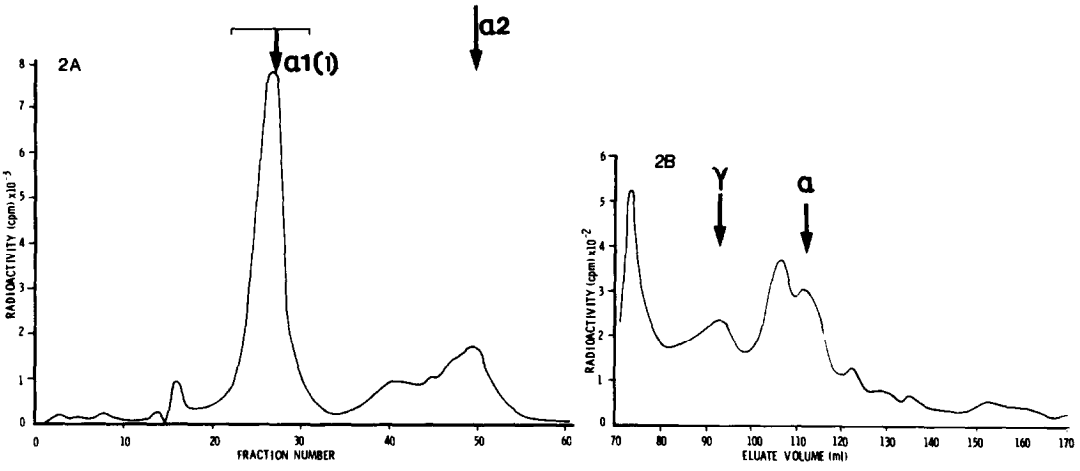

Figure 2. (A) CM cellulose chromatography of the precipitate at 2.1M NaCl obtained in the fractionation of radiolabelled cell layer collagens isolated after pepsin treatment (Expt. 2). Radioactivity in a 0.5 ml aliquot of each 10 ml fraction of the eluate was counted in 5 ml of scintillant (in polypropylene inserts) at 20% efficiency. The results relate to the cell layers pooled from six plates, each labelled with 0.5 mCi L [G-^{3}H] proline from 9-11d; one third of the total sample was chromatographed. (B) Gel filtration of α1(I) chains: Fractions 22-31 of the above chromatogram were pooled, dialyzed against 0.15M acetic acid, lyophilized and a portion then applied to a column of Sepharose CL-6B. Radioactivity in a 0.5 ml aliquot of each 2.5 ml fraction of the eluate was counted as in Fig. 1. The position of elution of chains as indicated was established by gel filtration of appropriate unlabelled samples.

Examination of radiolabelled collagens in the medium. To confirm the synthesis of interstitial types, radiolabelled collagens in the medium (from Expt 2) were subjected to thermal gelation to effect the separation of interstitial from basement membrane derived species (10) as described in Methods. Approx. 25% of the [^{3}H] hydroxyproline in the medium was in the fraction precipitated by dialysis against 0.02M Na_2HPO_4. Most (about 75%) of the remainder failed to precipitate (with appropriate carrier) even with 30% NaCl (16) and was considered to reflect possibly degradation products arising from the turnover of collagen (31,32). A pro/hyp radioactivity ratio of 1.5 following collagenase treatment appeared to preclude its derivation from Type IV collagen. The 30% NaCl-precipitated fraction revealed only a peak at the front on CM cellulose.

The initial gelation of the collagenous fraction precipitated by dialysis against 0.02M phosphate yielded a residue that represented approximately 40%

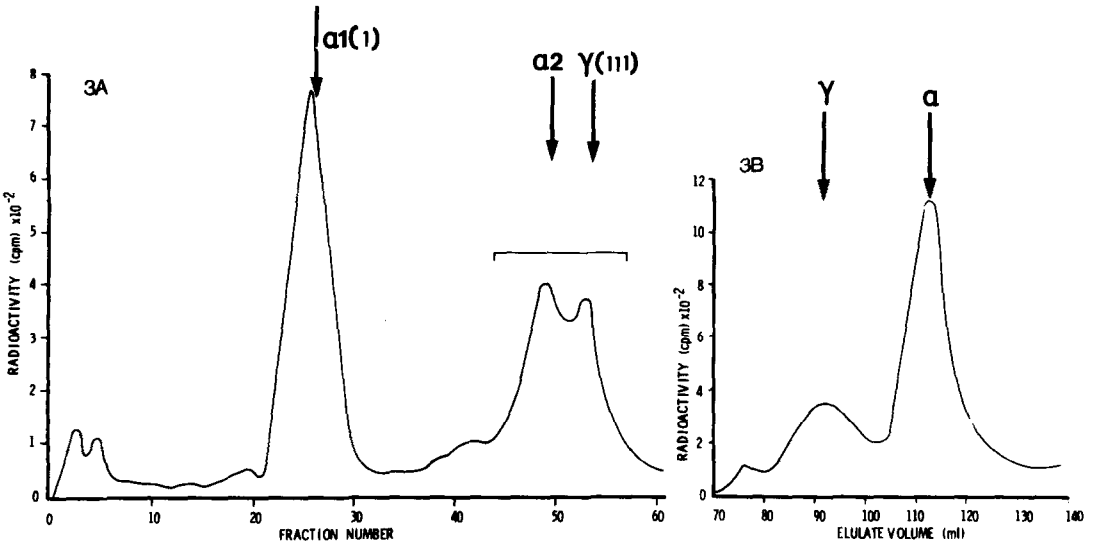

Figure 3. (A) CM cellulose chromatography of the radiolabelled collagens from the medium (Expt 2) precipitated by gelation at 37°: One quarter of the total sample was applied. The eluate was counted as in Fig. 1. (B) Fractions 44-57 of the above chromatogram were pooled, dialyzed and lyophilized and applied to a column of Sepharose CL-6B. The eluate was counted as in Fig. 1. The γ-chains are presumed to be derived from the Type III peak and the α-chains from the Type I α2 peak.

of the total radioactivity in this fraction. CM cellulose chromatography revealed the presence of both Types I and III collagens (Fig. 3). The Type III peak was of γ-chain size by gel filtration confirming its identification as Type III collagen (Fig. 3). The α1(I) and α2 peaks were in a ratio of approx 2:1 and were of α-chain size consistent with their derivation from Type I collagen. The supernatant revealed a major peak on CM cellulose in the vicinity of α1(I) chains. Since the gelation technique was subsequently found to precipitate only about a half of the unlabelled carrier collagen, it seemed likely that some labelled Type I must remain in the supernatant. Application of the modified gelation procedure (which was found to precipitate over 90% of interstitial collagens) yielded a fraction (representing about 75% of the radioactivity in the supernatant) that by CM cellulose contained mostly α1(I) and α2-chains with a small amount of Type III-chains. The α1(I): α2 ratio of about 4 suggested excess α1-chains and must it is thought reflect the

presence of α1(I) trimer. The final supernatant after the second gelation contained only about 5% of the initial [^{3}H]hydroxyproline and revealed on CM cellulose chromatography an absence of recognisable α-chains.

DISCUSSION: The cells used in this study were considered entirely endothelial in nature not only in view of the method of isolation employed but also from morphological appearance (see 3) and most importantly since they remained as monolayers at confluence. Our studies demonstrate that much (if not all) of the collagen synthesized by these cells in culture is interstitial in character. We do not consider this arises by contamination of our cultures with smooth muscle cells. In any event, the rate of incorporation of [^{3}H]proline by the latter (at least in our hands) is much lower than by endothelial cells and this would seem to preclude the possibility that interstitial collagens at the levels we observed could be derived from a low percentage of contaminating smooth muscle cells.

It is not possible to state at present whether the synthesis of interstitial as opposed to basement-membrane-related collagens represents a change in the pattern of collagen synthesis as a result of loss of phenotypic character arising through sub-culturing of the cells, as can occur for example with chondrocytes (19) or smooth muscle cells (33). We find a similar pattern of collagen synthesis whether the cells are labelled in the early "endothelial" phase or the later 'fibroblastic' phase (3).

The synthesis of interstitial collagens by endothelial cells in culture implies no simple distinction can be drawn between cells of epithelial type (34) producing basement-membrane collagens (IV and V) and the fibroblast and its differentiated forms producing interstitial collagens (I, II and III). Similarly smooth muscle cells have been shown to synthesize α[A] and α[B]-chains (Type V collagen) as well as Type I and III collagens (24) whilst embryonic chick corneal epithelium has been shown to synthesize interstitial collagen (35).

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