SYNTHESIS OF COLLAGENS TYPES I AND III BY PIG MEDIAL SMOOTH MUSCLE CELLS IN CULTURE

M.J. Barnes, L.F. Morton and C.I. Levene
MRC Connective Tissue Team, Dept. of Pathology, University of Cambridge, Tennis Court Road, Cambridge, U.K.

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Summary
Isotopic studies have indicated the synthesis by pig medial smooth muscle cells, in culture, of two species of collagen, both of which are known to occur in aorta, namely Types I and III. The latter was detected as a component of the cell layer that, following pepsin digestion, occurred as a γ-chain moiety that could be distinguished from Type I α-chains on CM cellulose or by gel chromatography and could be reduced to α-chain size by reduction of disulphide linkages.

Since the medial smooth muscle cell may play a central role in the genesis of arterial disease, especially atherosclerosis (see 1) we have been studying the behaviour of this cell in culture. It has been considered on the basis of radioautographic and electronmicroscopal studies, the site of synthesis of the extracellular connective tissue components in the media of the aorta (2,3,4). This view has now been substantiated in studies with isolated smooth muscle cells which have demonstrated by isotopic techniques the synthesis of collagen and elastin by these cells (5-8). Thus as regards collagen, Faris et al (7) have reported the synthesis of peptide-bound hydroxyproline, indicative of collagen synthesis, by rabbit medial smooth muscle cells in culture and Layman and Titus (8) have reported, additionally that cultured human smooth muscle cells synthesised collagen that proved to be of a single species only, namely Type I. Our own studies described below, also aimed at establishing the synthesis of collagen by smooth muscle cells in culture, indicate the synthesis of two species of collagen, Types I and III. This is of particular relevance in view of the known occurrence of both these collagens in aorta (9-14).

MATERIALS AND METHODS

Culture of smooth muscle cells. Smooth muscle cells were derived from explants of aortic media of young pigs, essentially as described by Ross (3).

The intima and immediate underlying part of the media were first dissected away from freshly collected aorta. A thin layer of the media so exposed was then removed for the preparation of explants. Cells obtained from explants were further subcultured three times. For the purposes of the present investigation cells obtained by this procedure (kindly provided by Mr. D.S. Leake of this Department) were grown at 37° in 60 mm. Falcon petri dishes in an atmosphere of 75% N_2/20% O_2/5% CO_2 as described by Levene & Bates (15) for 3T6 fibroblasts. Each dish contained approximately 65,000 cells in 4 ml. of the Dulbecco and Vogt modification of Eagle's MEM supplemented with 10% foetal calf serum. The medium which was changed twice weekly was supplemented with ascorbic acid at 50 µg/ml. after the 6th day. On the 9th and 11th days, fresh medium supplemented with ascorbic acid and containing 250 µCi of L-[G-^{3}H] proline was added to each plate. Medium was collected on the 11th and 12th days and cells harvested on the 12th day.

Pepsin digestion of the cell layer. The cell layer from two plates was dispersed in 10 ml. 0.5M acetic acid with a glass-teflon homogeniser. The dispersion was dialyzed several times against 0.5M acetic acid, containing initially proline at 1 mg/ml. After dialysis, a solution of unlabelled carrier collagen (50 mg. of acetic acid-extracted rat tail tendon collagen, salt-precipitated twice and dissolved in 10 ml. of 0.5M acetic acid) was added and the mixture then incubated with 20 mg. of pepsin (Sigma, 2x crystallized) for 18 h at 4° (9). Insoluble material was then removed by centrifugation. Prior to precipitation, the supernatant was further diluted with a solution of carrier collagen containing in this instance both types I and IIIcollagen prepared by pepsin digestion (9) of skin of 16 week old chickens. Following pepsin digestion, carrier collagen was precipitated from the pepsin-solubilized material by addition of sodium chloride to 0.9M. Approx. 100 mg. of precipitated material was dissolved in 35 ml. of 0.5M acetic acid and the solution added to the above supernatant. The total solution was then dialyzed successively against 0.15M acetic acid, water, 0.56 and 1.0M sodium chloride. Insoluble material was removed by centrifugation and the supernatant then dialyzed against a series of sodium chloride solutions of increasing molarity, as follows: 1.71, 1.97, 2.14 and 2.56M (16). Material precipitated at each molarity was collected by centrifugation. Precipitates were redissolved in 0.5M acetic acid, dialyzed against 0.15M acetic acid and then 1M urea in 0.05M sodium acetate, pH 4.8, prior to chromatography on CM cellulose.

Pepsin digestion of the medium. Medium containing L-[G-^{3}H] proline was administered on the 9th day and collected, from four plates in all, on the 11th day, dialyzed several times against 0.5M acetic acid, initially containing proline at 1 mg/ml., and then incubated with pepsin (Worthington's 2x crystallized) added at 100 µg/ml. for 6 h at 18° (17). The mixture was then freeze-dried and the residue dissolved in 0.1% SDS in 0.1M sodium phosphate buffer pH 7.4 at 45° for 15 min. prior to gel chromatography.

CM-cellulose chromatography. The constituent chains of collagen were separated by CM cellulose chromatography essentially as described by Piez et al (18) and Miller (19). The column (1.8x18cm) was equilibrated with the starting buffer, 1M urea in 0.05M sodium acetate pH 4.8, and after application of the sample, eluted with a linear gradient of sodium chloride from 0 - 0.1M in starting buffer, over a total volume of 800 ml. Chromatography was at 41°. Prior to application, the sample was equilibrated with starting buffer by dialysis and then denatured at 45° for 30 min. Elution of collagen chains was detected by counting the radioactivity in the eluate as previously described (20). Fractions were combined within peaks, dialyzed against 0.15M acetic acid at 4° and freeze-dried.

Gel chromatography. Samples were dissolved in 3 ml. of 0.1% SDS in 0.1M sodium phosphate buffer, pH 7.4 at 45° for 15 min. and examined on a column (70 x 2.5 cm) of Biogel A5m as described by Jimenez et al (21). Radioactivity in the eluate was measured as described above. Fractions were combined within peaks, dialyzed against water at 23°, 0.15M acetic acid at 4° and freeze-dried.

Reduction with β-mercaptoethanol. Samples were dissolved in 0.1M sodium phosphate buffer, pH 7.4, containing 2% SDS and 1% β-mercaptoethanol, incubated at 37° for 3 h (22), dialysed 3 x 1 h against 0.1% SDS in 0.1M sodium phosphate buffer pH 7.4 at 23° and then examined by gel chromatography as described above.

RESULTS

The cells employed in this investigation were considered smooth muscle cells not only on the grounds of their origin from media but also from their morphological appearance under the light microscope and from their ability to incorporate [^{3}H]-lysine into desmosines, indicative of the synthesis of elastin (C.I. Levene and M.J. Barnes, to be published).

The present study was concerned specifically with the ability of these cells to synthesize collagen. Their ability to synthesize Type III in addition to Type I collagen was investigated by subjecting the cell layer to pepsin digestion under conditions where the triple helical configuration of the collagen molecule is retained. This procedure serves to solubilize collagen normally rendered insoluble through cross-linking, by removal of the non-helical telopeptides through which cross-linkage occurs. Pepsin digestion is a particularly useful procedure for investigating Type III collagen since this collagen appears to be rapidly and almost in entirety rendered insoluble by the formation of disulphide linkages. Since digestion was carried out without prior extraction of the cell layer, the digest would also contain collagen normally soluble.

The pepsin digest was subjected to a differential salt precipitation procedure known to separate collagen Types I and III on the basis of the greater solubility of Type I collagen in salt solution. (9,16) The fraction of the digest precipitating at 1.71M sodium chloride contained negligible radioactivity and little material and was not investigated further. The

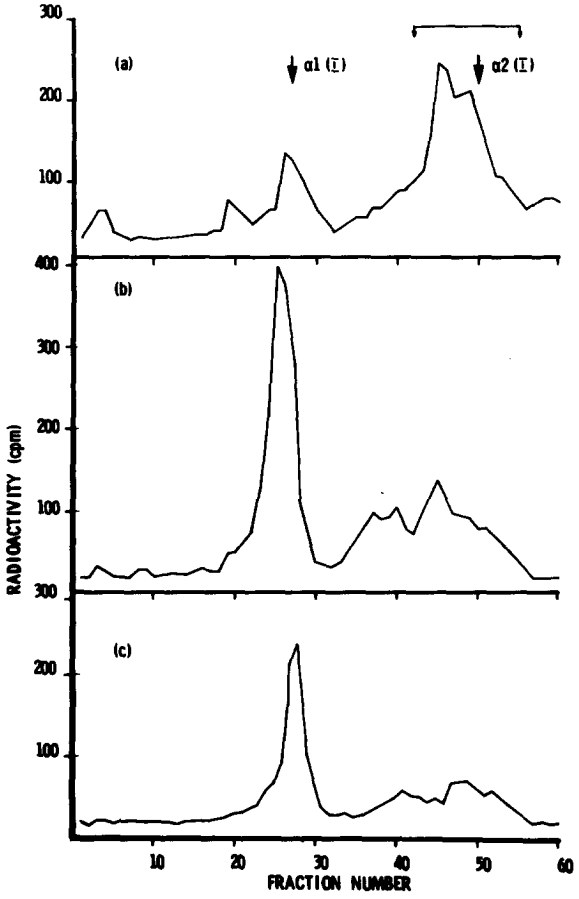

Figure 1. Pig aortic smooth muscle cells in culture: Chromatography on CM cellulose of the fractions obtained from the pepsin digest of the cell layer by precipitation at (a) 1.97 (b) 2.14 and (c) 2.56M sodium chloride. Conditions of chromatography are given in the text. The eluate was collected in 10 ml. fractions and the radioactivity in a 1 ml. aliquot of each counted as previously described (20).
The position of elution of collagen α1 (I) and α2 (I) chains was established by chromatography of rat tail tendon collagen. The fractions indicated in (a) were combined, dialyzed and freeze-dried prior to gel chromatography, results of which are shown in Fig.2.

fraction precipitating at 1.97M contained 44% of the total radioactivity precipitated. The bulk of the carrier collagen however (which consisted mostly of Type I) precipitated at 2.14 and 2.56M. The radioactivity in these two fractions was 38 and 18% respectively of the total radioactivity precipitated. Chromatography on CM cellulose of the three fractions

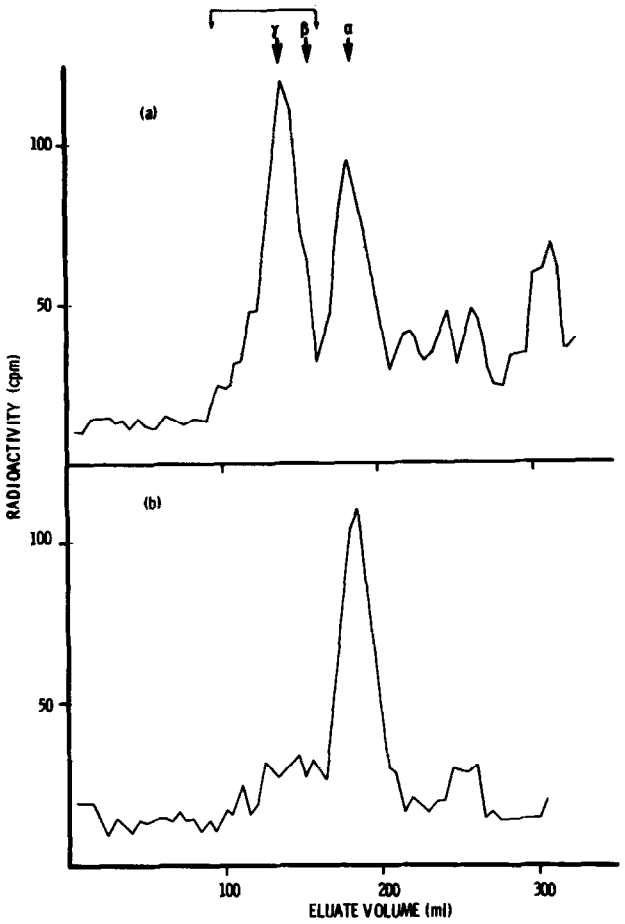

Figure 2. (a) Gel chromatography on Biogel A5M of the fractions indicated in Fig.1 (a). Conditions of chromatography are given in the text. The eluate was collected in 5 ml. fractions and the radioactivity in a 1 ml. aliquot of each measured (20). The γ-peak was considered to be γ-chains of Type III collagen and the α-peak to be α2 (I) chains. (b) The fractions indicated in Fig.2 (a) were combined, reduced with β-mercaptoethanol and re-examined on Biogel A5M as described in the text. Position of elution of collagen chains was established by chromatography of appropriate chains prepared for rat tail tendon collagen.

precipitating at 1.97, 2.14 and 2.56M sodium chloride, respectively, is shown in Fig.1. The fraction precipitating at 1.97M revealed a prominent peak eluting just before the α2 chains of Type I collagen, in a position to be expected for Type III collagen.(9,10,16) Gel chromatography revealed that this peak consisted of γ-chains (Fig.2). Further, reduction with β-mercaptoethanol converted the γ-chains almost completely to α-chains indicative of the presence of intramolecular disulphide linkages (Fig.2).

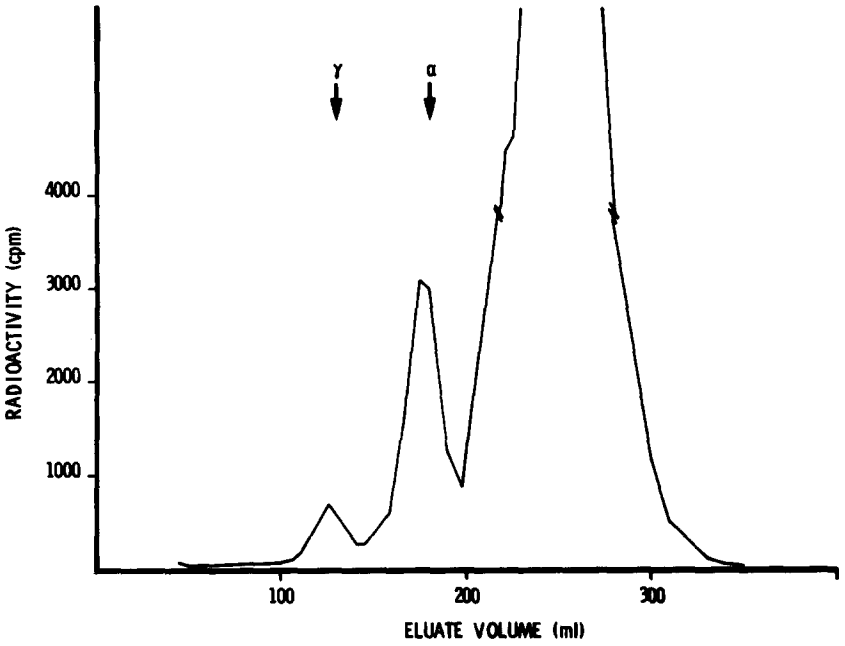

Figure 3. Gel chromatography of the medium, following pepsin digestion as described in the text. Conditions were as in Fig.2. Radioactivity was measured in 0.5 mls. of each fraction of the eluate. Results like those in Figs. 1 and 2, are at 23% efficiency of counting.

This behaviour is entirely consistent with the identity of this material as Type III collagen since the latter, isolated by pepsin digestion, is known to occur in the form of γ-chains, which can be converted to α-chains by reduction of disulphide linkages (9,10,16). These observations imply the presence of Type III collagen in the fraction precipitating at 1.97M sodium chloride. The fractions precipitating at 2.14 and 2.56M appeared from CM cellulose chromatography to contain mostly Type I collagen. Examination of the CM cellulose chromatograms indicated that Type III collagen represented in the region of 20-25% of the total collagen in the three fractions.

The medium was investigated for the possible presence of some soluble Type III collagen. The medium was subjected to pepsin digestion since it is known that collagen accumulating in the medium of cell cultures occurs mostly as the soluble precursor, procollagen. Conditions were those

described by Monson & Bornstein (17) for the purpose of converting procollagen to collagen. Examination of the pepsin digest by gel chromatography revealed a small peak in the position corresponding to γ-chains in addition to a prominent α-chain peak. (Fig.3). Reduction with β-mercaptoethanol converted the material of γ-chain size largely to α-chains. The presence of material of γ-chain size after pepsin digestion and its reduction to α-chain size by β-mercaptoethanol are compatible with the occurrence in the medium of some Type III procollagen.

DISCUSSION

The results of thisstudy provide further support that the medial smooth muscle cell can synthesize collagen and in addition provide evidence for the first time that this cell can produce more than one species of collagen. The occurrence of a component, in pepsin digests, that precipitated mostly before much of Type I collagen, its chromatographic behaviour on CM cellulose, eluting just prior to α2 (I) chains and the demonstration that it occurred as γ-chains that could be reduced to α-chain size by rupture of disulphide bonds all indicate its identity with Type III collagen. The reason for its precipitation mostly at 1.97 rather than as anticipated at 1.71M sodium chloride (16) is unclear but precise conditions for precipitation do appear to vary according to a number of factors and precipitation may not occur sometimes until even later than 1.97M sodium chloride (11). Our results indicated that of the collagen deposited in the cell layer, Type III represented about one-fifth and Type I the remaining four-fifths.

Several investigations have reported on the presence of both Type I and Type III collagen in aorta (9-14). The significance of the existence of two types within a single tissue is as yet unclear. The relative distribution of each type within the tissue may clearly be important in the proper functioning of the tissue and it is conceivable that a disturbance of this distribution may occur in the diseased state. Rauterberg and Bassewitz (11) report that Type

III represents about 40% of the total collagen in bovine aorta. Gay et al (14), by immunohistochemical techniques, have shown that it occurs in human aorta to the exclusion of Type I collagen in the area immediately underlying the internal elastic lamina. Its concentration in the media relative to that of Type I then diminishes as the adventitia is approached. McCullagh and Balian (13) have reported that human media contains mostly Type III collagen whereas in diseased intima, where invasion of smooth muscle cells from the media may occur, Type I predominates. Our studies indicate that the smooth muscle cell synthesizes both these types and clearly the relative proportion of each produced at any specific site may depend upon factors in the environment, factors which may alter under disease conditions. Ross (23) has shown that the proliferation of smooth muscle cells is sensitive to external factors in the media. We are now exploring the effect of various factors on the precise expression of these cells in relation to collagen synthesis. It is the operation of such factors that may explain the synthesis of both Types I and III collagen by the cells described in this study and the synthesis of only Type I collagen by the human cells studied by Layman and Titus (8).

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