Synthesis of Types I and III Procollagen and Collagen by Monkey Aortic Smooth Muscle Cells in Vitro†

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ABSTRACT: Analysis of pepsin-resistant proteins produced in culture by monkey aortic smooth muscle cells (SMC) indicates the synthesis of both types I and III collagen. As determined by carboxymethylcellulose chromatography and disc gel electrophoresis, SMC cultures synthesize more type III collagen than monkey skin fibroblast cultures; aortic adventitial cell cultures (a mixture of SMC and fibroblasts) synthesize an intermediate amount of type III collagen. Both types I and III procollagens can also be isolated from the culture medium of SMC and skin fibroblasts. The procollagens were separated by diethylaminoethylcellulose (DEAE-cellulose) chromatography and identified by electrophoresis and after cleavage with pepsin and cyanogen bromide. Quantitation of the procollagen by DEAE-cellulose chromatography suggests that 68% of the SMC procollagens and less than 10% of the skin fibroblast procollagens are type III. On the other hand, estimation of the proportions of collagen types secreted by cells, employing pepsin digestion of cell culture medium at 15 °C, leads to an underestimation of the amount of type III collagen relative to type I. SMC and fibroblasts may differ in their ability to convert type I procollagen to collagen as indicated by the observation that skin fibroblast culture medium contains both p_N and p_C collagen intermediates after 24 h, while cultures of SMC essentially lack the p_C collagen intermediates.

Collagen synthesis by aortic medial smooth muscle cells in vitro has been demonstrated both morphologically (Ross, 1973) and biochemically (Barnes et al., 1976; Faris et al., 1976; Layman and Titus, 1975; Leung et al., 1976). Layman and Titus (1975) reported that human fetal smooth muscle cells synthesize only type I collagen. Leung et al. (1976) and Barnes et al. (1976) (working with rabbit and pig aortic smooth muscle, respectively) reported that cultured cells synthesize both types I and III collagens, with a preponderance of type I. However, extraction of collagens from the medial layer of human aortas yielded approximately 70% type III and 30% type I collagen (McCullagh and Balian, 1975). Since the media of mammalian arteries contain only smooth muscle cells (Ross and Glomset, 1973), the available evidence suggests that smooth muscle cells in vitro express a different phenotype than in vivo in regard to the relative proportion of collagens synthesized.

In this report we characterize the collagens and procollagens synthesized by monkey aortic smooth muscle cells and demonstrate the synthesis of both types I and III collagen. Procollagen analyses yielded a proportion of the two proteins closely approximating that reported in aortic tissue.

Materials and Methods

Cell Cultures. Smooth muscle cells (SMC),[1] derived from explants of the medial layer of the thoracic aorta of the pigtail monkey *Macaca nemestrina,* were subcultured as reported previously (Ross, 1971). The cells were grown in Dulbecco-Vogt's modified Eagle's basal medium supplemented with 5% homologous blood serum (MBS) or 10% fetal calf serum (FCS). Cells were utilized after three to five trypsinizations.

Cell cultures derived from explants of monkey thoracic aorta adventitia and from monkey skin, grown by the same procedure as the SMC, were also analyzed for collagen synthesis.

Collagen Preparation. Nearly confluent cultures of SMC, adventitial cells, or skin fibroblasts were labeled for 24 h in serum-free medium containing 5–15 μCi/mL [2,3-^{3}H]proline and 50 μg/mL sodium ascorbate. The medium, plus one serum-free-medium rinse of the cell layer, was centrifuged at 15 500 rpm at 4 °C to remove cell debris and the supernatant was mixed with 10 mg of lathyritic rat skin collagen (LRSC) as carrier. The cell layers of the labeled cultures were scraped from the flask with a rubber policeman in serum-free medium and sonicated to disrupt the cells, and LRSC was added as carrier.

The medium and cell layer preparations were then treated separately, but identically, as follows. The proteins were precipitated with 20% ammonium sulfate at 4 °C, sedimented by centrifugation at 15 500 rpm, and suspended in and dialyzed against 0.5 M acetic acid. Pepsin (1% by weight of the carrier collagen) was added to the samples and digestion continued for 5 or 24 h at 15 °C. Pepsinization was terminated by raising the pH to 8.0 with 0.5 M NaOH. Samples were dialyzed against 0.1 M acetic acid and lyophilized. Aliquots were taken for disc gel electrophoresis in 5% acrylamide (Goldberg et al., 1972), unreduced in the presence of 100 mM iodoacetamide or reduced with 50 mM dithiothreitol. Dansylated chick collagen β_{11} components, α1(I) chains, and α1(I)CB-7 were used as internal standards. The gels were sliced and counted by standard methods. In other experiments aliquots were analyzed by electrophoresis on 5% acrylamide slab gels (Reid and Bielski, 1968) using the method of Laemmli (1970). Bands were visualized by fluorography according to the procedure of Bonner and Laskey (1974) and quantitated by densitometry

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[1]Abbreviation used: SMC, smooth muscle cell; MBS, monkey blood serum; FCS, fetal calf serum; LRSC, lathyritic rat skin collagen; DNS, dansyl; β-APN, β-aminopropionitrile; EDTA, ethylenediaminetetraacetic acid; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; MalNEt, *N*-ethylmaleimide; MF, microfibrillar; Tris, tris(hydroxymethyl)aminomethane.

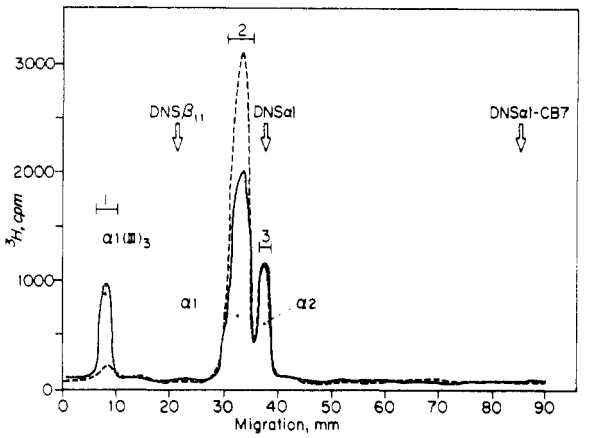

FIGURE 1: Electrophoresis in 5% acrylamide of pepsin-resistant radioactive proteins (24 h digestion) from the medium of SMC cell cultures. Before reduction (—), 20% of the labeled collagens are found in the large molecular weight peak representing unreduced type III collagen (peak 1). After reduction with dithiothreitol (- - -), this material comigrates with α1(I). The positions of dansylated type I collagen markers are indicated.

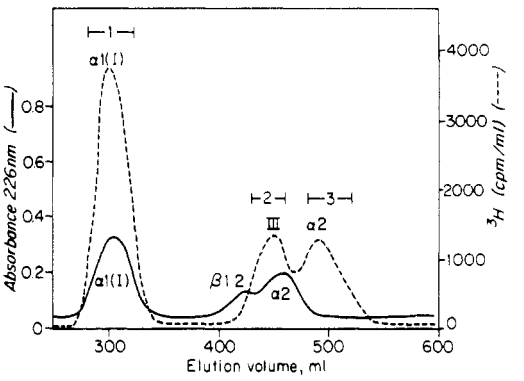

FIGURE 2: Pepsin-resistant radioactive proteins (24 h pepsin digestion) from the medium of SMC cell cultures (- - -) chromatographed with lathyritic rat skin carrier collagen (—) on CM-cellulose. Type III collagen (peak 2) is incompletely separated from the α2 chains of type I collagen (peak 3). Fractions represented by the horizontal bars were pooled for further analysis.

using a Quick Scan densitometer (Helena Laboratories, Beaumont, Texas).

The remainder of the sample was suspended in 0.04 M sodium acetate–4 M urea (pH 4.8) for carboxymethylcellulose (CM-cellulose) chromatography (Bellamy and Bornstein, 1971) using a linear gradient of 0–0.08 M NaCl over 600 mL. The effluent was monitored for absorbance at 226 nm and for radioactivity by standard spectrometric methods. Radioactive peaks were analyzed by disc or slab gel electrophoresis as described above.

Procollagen Preparation. Nearly confluent cultures of SMC or skin fibroblasts were rinsed with serum-free medium containing 50 μg/mL sodium ascorbate and 50 μg/mL β-aminopropionitrile (β-APN). Cells were incubated for 24 h in fresh medium containing the same additives as the rinse medium plus 15 μCi/mL [2,3-^{3}H]proline. Because nearly 90% of the labeled collagen available in the culture is found in the medium (unpublished data), procollagen analyses were restricted to this compartment. The medium was pooled with one rinse of the cell layer, and the following were added to prevent proteolysis: 50 mM EDTA, 1 mM phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$), and 50 μg/mL *N*-ethylmaleimide (MalNEt). All subsequent procedures were carried out at 4 °C. Cell debris was removed by centrifugation at 12 500 rpm. Medium proteins, precipitated with 20% ammonium sulfate and pelleted by centrifugation, were dialyzed against 50 mM Tris-HCl (pH 7.5), 4 M urea, 2.5 mM EDTA, 0.5 mM $PhCH_2SO_2F$, and 50 μg/mL MalNEt for chromatography on DEAE-cellulose (column size 10 × 1.6 cm), using a linear gradient of 0–200 mM NaCl over a total volume of 400 or 800 mL. Peaks were identified before and after pepsin treatment, by resolution in 5% acrylamide slab gels as described above. Additional peak material was suspended in 70% formic acid for cyanogen bromide (CNBr) cleavage (Miller et al., 1971). Peptides were resolved by gel electrophoresis in 12.5% acrylamide.

Preparation of Standard CNBr Peptides. Collagens were prepared from abdominal and/or thoracic aorta of four male *Macaca nemestrina* monkeys and from the skin of one male monkey. Collagens were extracted by pepsinization for 72 h at 4 °C and purified by repeated precipitation with 7.5% NaCl at acid pH and 20% NaCl at neutral pH. Types I and III collagens were differentially precipitated with 0.2 M increments of NaCl from 1.0 to 3.0 M NaCl as previously described (Chung and Miller, 1974). The precipitates at 1.8 and 2.0 M NaCl (predominantly type III collagen) and those at 2.4 and 2.6 M NaCl (predominantly type I collagen) were chromatographed on CM-cellulose using the same conditions described above. The resulting α1(I), α2, and α1(III) chains were cleaved with CNBr and peptides resolved by slab gel electrophoresis in 12.5% acrylamide and visualized by staining with Coomassie blue.

Results

SMC and Fibroblast Collagens. To determine the types of collagen synthesized by the SMC in culture, the labeled proteins from the culture medium were cleaved with pepsin to remove the nonhelical portion of the collagen precursor and characterized by electrophoresis in 5% acrylamide and by chromatography on CM-cellulose. After disc gel electrophoresis, three peaks of radioactivity were observed (Figure 1). Before reduction peak 1 migrated in a position indicating a size larger than the dansylated β_{11} internal standard. Peaks 2 and 3 migrated in the region of the dansylated α1(I) marker and appeared in a ratio of 2:1, the expected ratio of α1(I) to α2 chains in type I collagen. The migration of peaks 2 and 3 was not altered by reduction. Since dansylation of α1 results in a slight increase in its migration rate (Monson and Bornstein, 1973), unreduced peaks 2 and 3 were identified as α1(I) and α2, respectively. On reduction, the large molecular weight peak 1 material disappeared and peak 2 was correspondingly augmented. This observation is consistent with the identification of peak 1 as trimeric, disulfide bonded, type III collagen, which can be converted to α1(III) chains on reduction (Chung and Miller, 1974).

When pepsin-resistant medium proteins were chromatographed on CM-cellulose with LRSC carrier collagen, three peaks of radioactivity resulted (Figure 2). In order of elution, the peaks were identified as α1(I), type III collagen, and α2, based on the elution positions from CM-cellulose of corresponding human collagens (Chung and Miller, 1974; Chung et al., 1974; Epstein, 1974; Trelstad, 1974). Like the human α2 chains, monkey α2 eluted after the α2 chains of rat collagen (Layman et al., 1971). Radioactive peaks were pooled as indicated in Figure 2 and analyzed by electrophoresis. CM-cellulose peaks 1 and 3 migrated in the same positions before and after reduction, but peak 2 migrated before reduction as high-molecular-weight material and after reduction in the position of α chains (data not shown). Peak 2 thus contained

TABLE I: Estimation of the Amount of Culture Medium Type III Collagen, Relative to Type I, Using Different Methods of Quantitation.[a]

Method	% type III (SMC cultures)	% type III (skin fibroblast cultures)
CM-cellulose chromatography (24-h pepsin digestion)	20	10
Disc gel electrophoresis (24-h pepsin digestion)	20	ND[b]
CM-cellulose chromatography (5-h pepsin digestion)	55	ND
Densitometry of fluorograms, slab gel electrophoresis (5-h pepsin digestion)	30[c]	ND
DEAE-cellulose chromatography (Procollagens)	68	10
Densitometry of fluorograms, slab gel electrophoresis of CNBr peptides (procollagens)	38[d]	9

[a]Methods described in text. [b]Not determined. [c]Densitometry reproducibly yielded lower estimates of type III collagen than did CM-cellulose chromatography of the same preparations. [d]This value is based on comparisons of the density of the band representing α1(I)CB-7 plus α2CB-4 with the bands representing either α1(III)-CB-5 or α1(III)CB-3. Densitometry yielded lower estimates of type III procollagen than did DEAE-cellulose chromatography of the same preparations.

disulfide-bonded α chains, in agreement with its identification as type III collagen.

When the proportion of labeled types I and III collagen in the medium of SMC is estimated from CM-cellulose chromatography of material pepsinized for 24 h, assuming that the radioactivity in peak 2 (Figure 2) represents type III collagen, it appears that monkey SMC secrete about 20% type III and 80% type I collagen (Table I). A similar conclusion is reached when the proportion of type III collagen is estimated from disc gel electrophoresis, assuming that the high-molecular-weight peak (peak 1, Figure 1) represents type III collagen.

After labeling with [^{3}H]proline for 24 h, most (nearly 90%) of the collagen-associated radioactivity was found in the culture medium. Analyses of cell layer collagens after 24-h pepsin treatment (not shown) also indicated the presence of both types I and III procollagens, with type I predominating, as in the medium. The proportion of collagen types in the medium and the cell layer was not altered by growth in FCS or MBS.

The medium from cell cultures derived from explants of monkey aortic adventitia and monkey skin was analyzed by the same procedures used for SMC (not shown). CM-cellulose chromatograms and acrylamide electrophoretograms were qualitatively identical with those resulting from SMC. However, estimates of the proportions of types I and III collagens for these cell cultures, after 24-h pepsin treatment, indicated that monkey adventitial cells (probably a mixture of smooth muscle and fibroblasts) secrete about 17% type III, and monkey skin fibroblasts secrete about 10% type III collagen.

SMC Procollagens. Three major peaks were observed after DEAE-cellulose chromatography of [^{3}H]proline-labeled SMC culture medium proteins, protected from degradation by protease inhibitors (Figure 3). Previous studies with human skin fibroblasts suggested that two of these peaks correspond to types I and III procollagen, with type I procollagen eluting prior to type III (Lichtenstein et al., 1975). In the present study the peaks were characterized by (1) electrophoresis and (2)

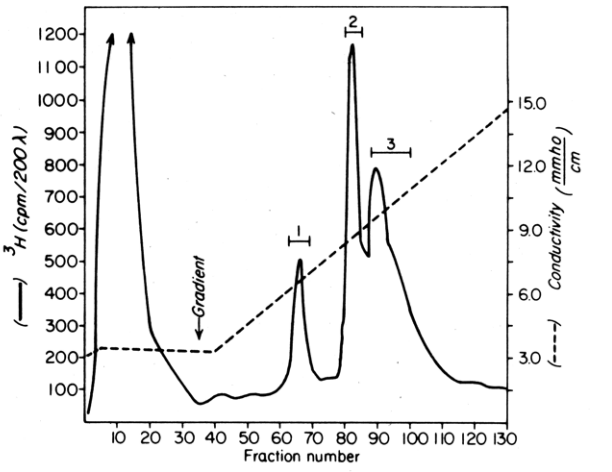

FIGURE 3: DEAE-cellulose chromatogram of radioactive medium proteins of SMC cell cultures. Major components of peaks 1, 2, and 3 are respectively type I procollagen, type III procollagen, and the MF protein. The peak eluting prior to the start of the gradient consists largely of unidentified noncollagenous components.

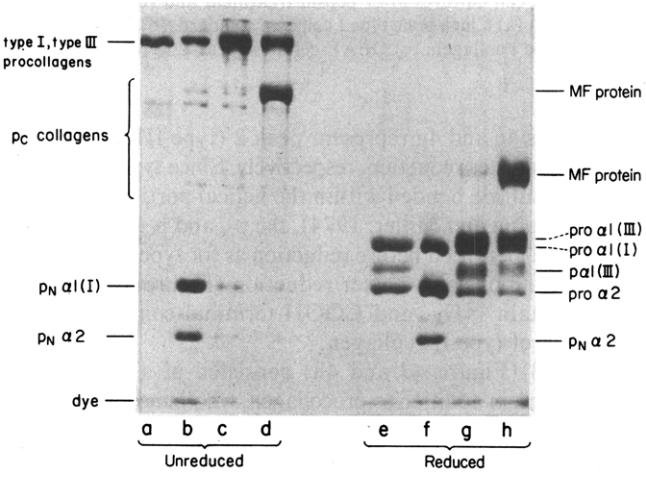

FIGURE 4: Electrophoresis in 5% acrylamide of DEAE-cellulose peaks from SMC cell cultures before and after reduction with dithiothreitol. Unreduced: (a) chick bone type I procollagen standard; (b) peak 1, type I procollagen and p_N collagen; (c) peak 2, predominantly type III procollagen; (d) peak 3, MF protein and procollagens. Reduced: (e) chick bone type I procollagen standard; (f) peak 1, procollagen and p_N collagen; (g) peak 2, predominantly type III procollagen and p-collagen intermediates; (h) peak 3, MF protein and procollagens.

cyanogen bromide digestion followed by electrophoresis in 12.5% acrylamide, with comparison to CNBr peptide standards prepared from monkey skin and aorta.

Fractions from DEAE-cellulose chromatography were pooled as indicated in Figure 3 and analyzed by electrophoresis before and after reduction with dithiothreitol (Figure 4). Before reduction, peak 1 (Figure 4b) exhibited three major bands: (1) the slowest migrating type I procollagen, a protein which is disulfide-bonded in the COOH-terminal region of the molecule (Byers et al., 1975; Fessler et al., 1975; Olsen et al., 1976); (2) $p_N\alpha1(I)$ chains; and (3) $p_N\alpha2$ chains. (P_N chains contain the NH_2- but lack the COOH-terminal extension.) The faint bands between the procollagen and $p_N\alpha1(I)$ bands probably represent the intermediates in procollagen conversion representing the stepwise scission of the COOH-terminal extension (p_C collagen intermediates) (Davidson et al., 1975). After reduction (Figure 4f), peak 1 generated predominantly proα and $p_N\alpha$ chains. Peak 1 thus contained both type I procollagen and a substantial fraction of type I p_N collagen.

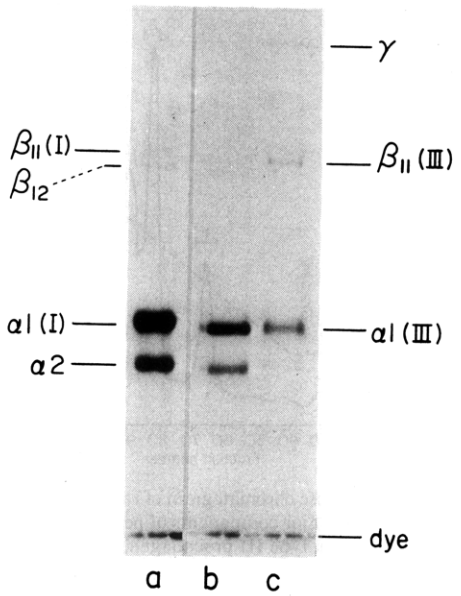

FIGURE 5: Electrophoresis in 5% acrylamide of DEAE-cellulose peaks from SMC cell cultures after pepsin treatment and reduction with dithiothreitol. (a) Chick bone type I collagen standard; (b) DEAE-cellulose peak 1, type I collagen; (c) DEAE-cellulose peak 2; type III collagen.

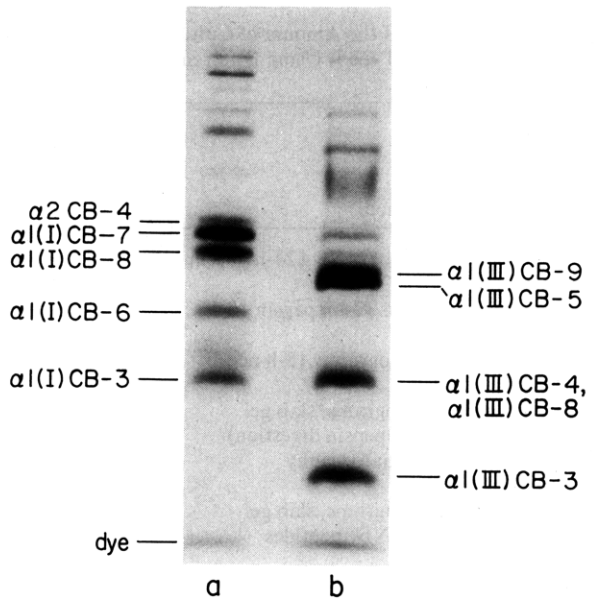

FIGURE 6: Electrophoresis in 12.5% acrylamide of DEAE-cellulose procollagen peaks from the culture medium of SMC after cleavage with CNBr and reduction with dithiothreitol. (a) DEAE-cellulose peak 1, type I procollagen; (b) DEAE-cellulose peak 2, type III procollagen.

Figures 4c and 4g represent peak 2 (type III procollagen) before and after reduction, respectively. Since type III collagen is also disulfide bonded within the helical portion of the molecule (Chung and Miller, 1974), the p_N and p_C intermediates cannot be identified before reduction as for type I. The middle major band of peak 2 after reduction (Figure 4g, pα1(III)) may contain NH_2- and COOH-terminal-containing intermediates of type III collagen.

Peak 3 (Figure 4d and 4h) consisted of a single major component, in addition to procollagen, whose molecular weight after reduction is larger than that of proα chains (Figure 4h). This protein may be related to the microfibrillar protein (MF protein) of the elastic fiber (Muir et al., 1976) or to a cell surface glycoprotein (Yamada and Weston, 1974; Keski-Oja et al., 1976).

Peaks 1, 2, and 3 from DEAE-cellulose were cleaved with pepsin, reduced, and analyzed by electrophoresis (Figure 5). Peak 1 yielded two major bands corresponding to α1(I) and α2 chains, and two minor bands in the position of β_{11} and β_{12} components (Figure 5b); peak 2 yielded a single major band in the α chain position, which we can identify as α1(III), and a minor band in the position of β_{11}(III) (Figure 5c); peak 3 (MF protein) is digested by pepsin to low-molecular-weight peptides.

The electrophoretic patterns of the major CNBr peptides of peaks 1 and 2 from DEAE-cellulose chromatography are shown in Figure 6. The peptide patterns are similar to those resulting from purified types I and III collagen extracted from monkey aorta or monkey skin (data not shown). Peak 1 contained type I collagen-specific CNBr peptides (Figure 6a) and peak 2 contained peptides specific for type III collagen (Figure 6b). The CNBr peptide patterns for monkey types I and III collagen are similar to those previously published for rat and human collagen CNBr peptides (Byers et al., 1974; Rojkind and Martinez-Palomo, 1976). Specific monkey type I and type III peptides were identified by relating the positions of electrophoretic migration to the molecular weights for corresponding human peptides (Click and Bornstein, 1970; Chung and Miller, 1974; Epstein and Munderloh, 1975).

Since types I and III procollagen can be successfully separated on DEAE-cellulose, this chromatographic procedure can be used to estimate the proportion of these two collagen precursors in cell cultures (Lichtenstein et al., 1975). We estimate that SMC cultures secrete into the medium about 68% type III procollagen and about 32% type I procollagen during a 24-h period (Table I). This estimate is the mean of determinations made on SMC cell cultures derived from five different animals (the range for the proportion of type III procollagens was 60 to 73%). The same proportion of procollagens was found in the medium when the cells were grown prior to labeling in MBS or FCS.

Skin Fibroblast Procollagens. The medium of monkey skin fibroblast cell cultures was analyzed for procollagen after labeling with [^{3}H]proline in procedures identical with those used for the SMC. DEAE-cellulose chromatography of medium procollagens revealed two peaks, the first of which predominated (Figure 7). Electrophoresis of these peaks indicated that peak 1 contained type I procollagen and intermediates, and peak 2 contained a mixture of type III procollagen and the MF protein (Figure 8). These fractions were further characterized after CNBr digestion (Figure 9). If, as for the SMC, one estimates the proportion of procollagens by DEAE chromatography, more than 90% of the fibroblast medium procollagens are type I (Table I). The relative amounts of types I and III procollagen were also quantitated by densitometric scans of slab gel fluorographs of CNBr peptides (Figure 9). Comparison of the densities of type-specific peptides, corrected for molecular weights, indicated that more than 90% of the fibroblast medium procollagens were type I (Table I).

Type I procollagen predominated in fibroblast cell culture medium, while type III predominated in the SMC cultures. In addition, there were two other noteworthy differences in the medium proteins of the two types of cell cultures. (1) As indicated in Figure 4, SMC cell culture medium contained a large amount of type I p_N collagen and little p_C collagen. In contrast, fibroblast cell culture medium contained both p_N and p_C collagen intermediates (Figure 8). (2) There is more MF protein, relative to procollagens, in the SMC cell cultures than

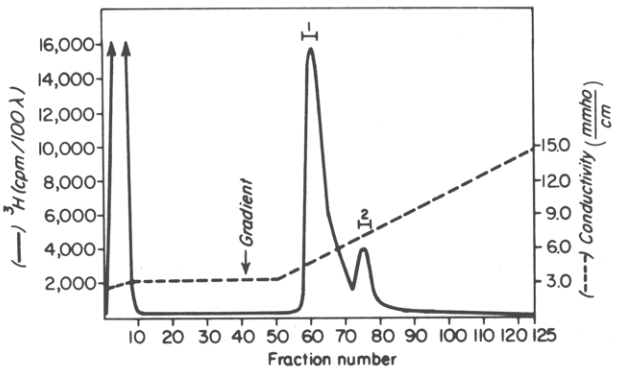

FIGURE 7: DEAE-cellulose chromatogram of radioactive proteins in the culture medium of skin fibroblasts. Peak 1 contains type I procollagen and peak 2 contains a mixture of type III procollagen and the MF protein.

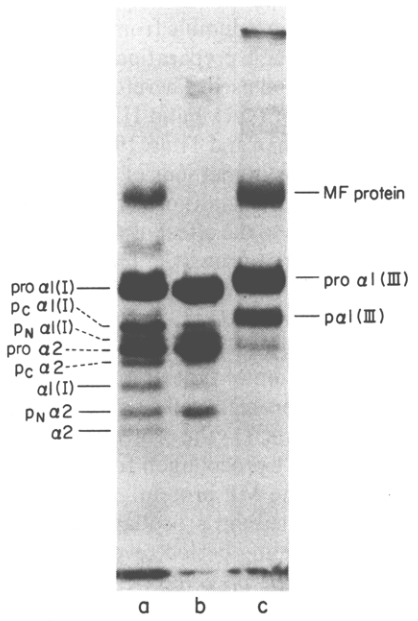

FIGURE 8: Electrophoresis in 5% acrylamide of the DEAE-cellulose peaks shown in Figure 7 after reduction with dithiothreitol. (a) Medium proteins prior to fractionation on DEAE-cellulose; (b) peak 1, type I procollagen; (c) peak 2, type III procollagen and the MF protein.

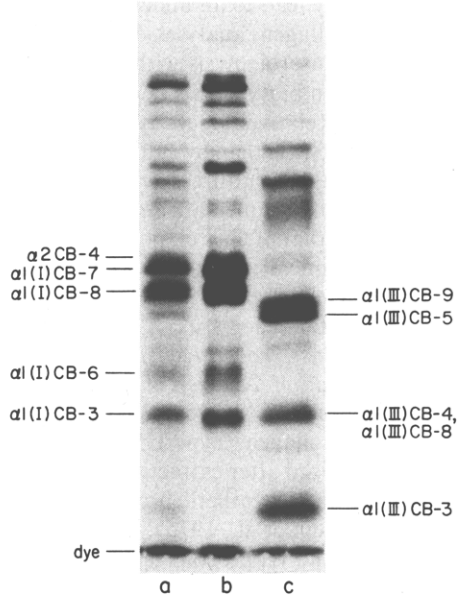

FIGURE 9: CNBr peptide patterns of dithiothreitol-reduced fractions obtained by DEAE-cellulose chromatography of skin fibroblast culture medium (Figure 7). Electrophoresis was performed in 12.5% acrylamide. (a) Medium proteins prior to fractionation on DEAE-cellulose. Material prepared in this way was scanned to quantitate the collagen types by densitometry of type-specific peptides (see Table I); (b) DEAE-cellulose peak 1, type I procollagen; (c) DEAE-cellulose peak 2, type III procollagen and the MF protein. CNBr cleavage products of the MF protein do not contribute substantially to the peptide pattern of type III procollagen produced under these conditions.

in cultures of skin fibroblasts. If the ammonium sulfate precipitate, prior to its fractionation of DEAE-cellulose (Figure 8a), is analyzed densitometrically after electrophoresis, the MF protein represents about 10% of the large molecular weight material in the fibroblast culture medium and 25–30% in the SMC culture medium.

Quantitation of Procollagen Types: Effect of Pepsin Digestion. The proportion of types I and III procollagens found in the medium of SMC cell cultures as estimated by DEAE-cellulose chromatography (68% type III) is significantly different from the proportion estimated by CM-cellulose chromatography or disc gel electrophoresis of the pepsin-treated proteins (20% type III). The major differences between the two types of analyses were the preparation of the material for procollagen analysis which involved the use of β-APN in the labeling medium and protease inhibitors in the harvesting medium, and the limited proteolysis by pepsin used to generate material for collagen analysis. Since it was determined that the preparation procedure for procollagen analysis was not responsible for the observed differences in the estimated proportions of types I and III collagen, we investigated the effects of pepsin proteolysis on the relative yields of the two proteins.

Types I and III procollagen synthesized by SMC were isolated by DEAE-cellulose chromatography and separately incubated with pepsin. Results indicated a 50% greater loss of type III than type I procollagen under these conditions. Further, reduction of the time of pepsin treatment from 24 to 5 h resulted in increased relative yields of type III collagen (from 20 to 55%) when the collagen types were quantitated by CM-cellulose chromatography (Table I). The milder 5-h pepsin treatment also resulted in relatively more type III collagen when collagen types were quantitated after electrophoresis (from 20 to 30%). It should be noted that electrophoretic quantitation after 24-h pepsinization was by counting sliced disc gels, while quantitation after 5-h pepsinization was by densitometry of slab gels (Table I).

Although various quantitation methods can result in markedly different estimations of the proportions of collagen types, harsher pepsin treatment routinely resulted in lower relative yields of type III collagen than did mild or no pepsin treatment when quantitated by the same procedure (summarized in Table I).

Discussion

SMC and Fibroblast Collagens. Previous studies of the collagens produced by aortic SMC in cell culture, which have utilized pepsin-treated proteins, indicated the synthesis of either type I collagen (Layman and Titus, 1975) or of both types I and III with a preponderance of type I collagen (Barnes et al., 1976; Leung et al., 1976). Using techniques similar to those of Barnes et al. (1976) in their study of pig aortic SMC, we recorded nearly the same proportions of types I and III collagen synthesized by monkey SMC as did these workers (about 20% type III). Although, as discussed below, the use of pepsin-

treated material may lead to an underestimation of type III relative to type I collagen, analyses of cell culture medium proteins after limited proteolysis with pepsin did indicate a difference in collagen biosynthesis between SMC and fibroblast cell cultures. Relatively more type III collagen is produced by cultures of SMC than by cultures of skin fibroblasts, and aortic adventitial cell cultures (a mixture of SMC and fibroblasts) produce an intermediate amount. This observation correlates well with that of Gay et al. (1975) who identified, by immunohistochemical techniques, increased amounts of type I collagen relative to type III in the adventitia as compared with the media of human aortas.

SMC and Fibroblast Procollagens, Pepsin Treatment and Methods of Quantitation. Analysis of the procollagens found in the culture medium of SMC revealed the presence of both types I and III procollagen. Type III procollagen has been previously characterized after extraction from rat skin (Byers et al., 1974) and as a biosynthetic product of human skin fibroblasts (Lichtenstein et al., 1975). Here we identified the presence of types I and III procollagens in SMC culture medium by (1) elution from DEAE-cellulose (Lichtenstein et al., 1975), (2) demonstration by electrophoresis of pro α1(I), pro α2, and pro α1(III) chains, (3) identification by electrophoresis of α1(I), α2, and α1(III) chains after pepsin treatment, and (4) demonstration of CNBr peptide patterns typical of types I and III collagens extracted from skin and aorta.

According to the suggestion of Lichtenstein et al. (1975), the procollagens eluted from DEAE-cellulose were used to quantitate the proportions of types I and III procollagen synthesized by cells in vitro. Unlike human (Lichtenstein et al., 1975) and monkey skin fibroblasts which synthesize a great excess of type I procollagens, aortic SMC cultures synthesize predominantly type III procollagen (68%) and a lesser amount of type I (32%). This proportion of the two collagen types correlates well with the observation that, of the collagens extractable from human aortic media, 70% are type III (McCullagh and Balian, 1975).

Because data using material obtained from SMC by pepsin treatment at 15 °C demonstrated a reduced amount of type III collagen, compared with observations of smooth muscle-containing tissues, we suggest that quantitation of the procollagens secreted by cultured cells is a more reliable measure of their biosynthetic activity. Further, we offer evidence suggesting that cell culture-derived type III procollagen, when cleaved with pepsin, may be more labile to enzymatic digestion than type I procollagen. Observations supporting this suggestion include the following: (1) quantitation by DEAE-cellulose chromatography of procollagen types (not treated with pepsin) gives the highest estimates of type III, (2) pepsinization of separated types I and III procollagens results in a relatively greater loss of type III, and (3) milder pepsin treatment (5-h incubation) results in greater estimates of type III than harsher pepsinization conditions (24-h incubation). Miller et al. (1976) recently reported that type III collagen contains a trypsin-sensitive site. Although we have not made a systematic search for large molecular weight fragments of type III collagen released by pepsin, no peptide as large as the 71 000-dalton tryptic fragment isolated by Miller et al. (1976) was identified. Perhaps alterations of the type III molecule after initial treatment with pepsin render it susceptible to further degradation such that only smaller peptide products result. Alternatively, a fraction of the pepsin-treated type III collagen may lose the COOH-terminal interchain disulfide bonded region and migrate, in gel electrophoresis, with α1(I) chains.

Since digestion with pepsin is the established method for extraction of type III collagen from tissues (Chung and Miller, 1974; Chung et al., 1974; Epstein, 1974; Trelstad, 1974), the susceptibility of type III collagen to pepsin degradation suggests that estimations of the type III content of tissues may be low. That is, although extraction of tissues with pepsin is essential for workable yields of type III collagen, pepsin treatment may lead to relatively greater loss of type III than type I collagen. It is possible, however, that pepsin digestion alters quantitation of collagen types more markedly in cell culture medium where the quantity of protein is low and not in tissues where there is a large amount of pepsin-susceptible protein. Furthermore, extraction of tissues with pepsin at 4 °C, as is commonly performed (Chung and Miller, 1974), may reduce the selective degradation of type III collagen.

We have tried a variety of methods to quantitate types I and III collagens synthesized in cell culture (summarized in Table I) and have found that none is entirely satisfactory. Although most procedures are reproducible from experiment to experiment, estimates on a single preparation may vary significantly depending on the method used. Therefore, discrepancies in the reported proportions of types I and III collagens synthesized by SMC in vitro (Layman and Titus, 1975; Barnes et al., 1976) may reflect differences in methods of quantitation as well as differences in cell culture conditions or in the species or tissue of origin. With regards to the effect of the cell culture medium, we have established that growth in fetal calf serum or adult monkey serum did not alter the proportion of collagen types synthesized by monkey SMC.

SMC vs. Fibroblast Cell Cultures. In addition to the difference in the proportion of types I and III procollagen synthesized, SMC and fibroblast cell cultures differed in at least two other major respects: (1) the proteolytic activities involved in conversion of type I procollagen to collagen, and (2) the relative synthesis of the MF protein.

Analysis of SMC medium procollagens intermediates isolated after a 24 h labeling period revealed largely type I p_N collagen, with very little p_C collagen. Similarly prepared fibroblast cell cultures, however, demonstrated both p_N and p_C intermediates. The observation of p_N collagen in one type of cell culture and both p_N and p_C in another is in accord with suggestions from other work that different enzymes cleave the NH_2- and the COOH-terminal regions in type I procollagen (Davidson et al., 1975). The data further imply that fibroblasts in cell culture have more amino-terminal cleaving activity than do SMC. Davidson et al. (1975) suggested that cleavage occurs first in the NH_2-terminal region of type I procollagen in the normal conversion process in chick cranial bone in organ culture. The presence of a large p_N collagen component in SMC cultures suggests that the NH_2-terminal need not be cleaved first and that the sequence of conversion may be cell or tissue specific.

In addition to the procollagens, another large molecular weight protein is found in the medium of both monkey SMC and skin fibroblasts. This protein is designated the "MF protein" because it may be related to the microfibrillar protein of the elastic fiber (Muir et al., 1976). More MF protein, relative to procollagen, is synthesized by SMC (25–35%) than by skin fibroblasts (10%). Large numbers of elastic fibers are found in the aortic media. Although the identity of the MF proteins with the morphological entity called the elastic fiber microfibril remains to be established, the observation of increased synthesis of MF protein by media-derived cells is at least consistent with this hypothesis. The MF protein found in the medium of these cell cultures may also be related to a cell surface glyco-

protein of similar size and properties (Yamada and Weston, 1974; Keski-Oja et al., 1976). The relationship between these extracellular proteins is presently under investigation.

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