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# Collagen Synthesis by Human Amniotic Fluid Cells in Culture: Characterization of a Procollagen with Three Identical Pro$\alpha$1(I) Chains†

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With the technical assistance of Theresa Canfield

ABSTRACT: Second trimester human amniotic fluid cells synthesize and secrete a variety of collagenous proteins in culture. F cells (amniotic fluid fibroblasts) are the most active biosynthetically and synthesize predominantly type I with smaller amounts of type III procollagen. Epithelioid AF cells (the predominating clonable cell type) synthesize a type IV-like procollagen and a procollagen with three identical pro$\alpha$ chains, structurally and immunologically related to the pro$\alpha$1 chains of type I procollagen. The latter procollagen, when cleaved with pepsin and denatured, yields a single non-disulfide-bonded $\alpha$ chain that migrates more slowly than F cell or human skin $\alpha$1(I) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis but coelutes with these chains from carboxymethylcellulose. The major cyanogen bromide produced peptides demonstrate a similar behavior relative to peptides derived from $\alpha$1(I). The collagen is characterized by an increased solubility at neutral pH and high ionic strength, relative to type I collagen. The amino acid composition of the pepsin-resistant $\alpha$ chain is essentially identical with that of human $\alpha$1(I), except for marked increases in the content of 3- and 4-hydroxyproline and hydroxylysine. Preliminary experiments suggest that these increased posttranslational modifications are responsible for the unusually slow migration of this collagen and its cyanogen bromide peptides on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The procollagen has, therefore, been assigned the chain composition [pro$\alpha$1(I)]$_3$. Like type I procollagen, [pro$\alpha$1(I)]$_3$ undergoes a time-dependent conversion, in the medium and cell layer, to procollagen intermediates and $\alpha$ chains. The production of [pro$\alpha$1(I)]$_3$ probably reflects the state of differentiation and/or embryologic derivation of AF cells rather than a characteristic of the fetal phenotype, since F cells do not synthesize significant amounts of the procollagen.

Fetal cells cultured from amniotic fluid have been utilized for the prenatal diagnosis of a variety of cytogenetic and inherited metabolic disorders (Milunsky, 1973). There has, however, been confusion in the literature regarding the morphologic identification of amniotic fluid cells and very little is known about their embryologic origin or state of differentiation. Although the presence of epithelioid cells in amniotic fluid has been noted, the cells emerging from long-term mass cultures were most frequently described as fibroblasts or fibroblast-like. Recent studies by Hoehn et al. (1974, 1975) have demonstrated that there are at least three distinct cell types which may be cloned from second trimester human amniotic fluid and subsequently propagated in culture. The cells were designated as E (epithelial), F (fibroblastic), and AF (cells with intermediate morphology and the predominating clonable cell type). These cell types have been distinguished on the basis of their clonal and cellular morphology (Hoehn et al., 1974, 1975), ultrastructure, growth characteristics (Hoehn et al., 1974), and most recently by their secretory products (Priest et al., 1977; Megaw et al., 1977; Crouch et al., 1978).

Relatively little is known about collagen production by amniotic fluid cells. Macek et al. (1973) presented evidence that mass cultures of cells with "uniform fibroblast morphology" synthesized hydroxyproline-containing proteins. Subsequently, Hurych et al. (1976) demonstrated the synthesis of type I collagen by long-term cultures of "amniotic fluid fibroblasts". Most recently, Priest et al. (1977) examined the collagens synthesized by clones and mass cultures of F and AF cells. F cells were found to synthesize type I collagen on the basis of chromatography on CM[1]-cellulose. However, AF cells

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[1] Abbreviations used: DMEM, Dulbecco-Vogt modified Eagle's medium; FCS, fetal calf serum; PBS, calcium- and magnesium-free phosphate-buffered saline; Tris-saline, 0.15 M NaCl, 0.05 M Tris-HCl (pH 7.5); $NaDodSO_4$ sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; $\beta$-APN, $\beta$-aminopropionitrile fumarate; MalNEt, *N*-ethylmaleimide; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; BSA, bovine serum albumin (fraction V); DEAE, diethylaminoethyl; CM, carboxymethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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were found to synthesize a higher proportion of collagen chains eluting with $\alpha$1(I) relative to $\alpha$2. Furthermore, the collagen preparations had relatively high ratios of 3-hydroxyproline to 4-hydroxyproline. On the basis of these observations, Priest et al. suggested that the major collagen synthesized by AF cells is related to type IV collagen.

In the present paper, we describe the collagens synthesized by human AF and F cells in culture. Specifically documented are (1) the biosynthesis by euploid AF cells of a procollagen with three identical pro$\alpha$1 chains, biochemically and immunologically related to the pro$\alpha$1 chain of type I procollagen, and (2) the conversion of this procollagen in culture to p-collagen intermediates[2] and collagen. The protein, which behaves differently from type I procollagen on $NaDodSO_4$-polyacrylamide gel electrophoresis and ion-exchange chromatography, is unusual in that prolyl and lysyl residues are hydroxylated to a much greater extent than previously reported for type I collagen or "type I trimer".

## Experimental Procedures

### *Materials*

Powdered DMEM, penicillin G, and streptomycin sulfate were purchased from Grand Island Biological Co., Inc.; trypsin (1:250) was from ICN Pharmaceutical, Inc.; bacterial collagenase (Form III) was from Advance Biofactures; pepstatin A was from the Protein Research Foundation (Osaka, Japan); BSA and rabbit IgG were from Miles Laboratories. CNBr was from Eastman Kodak. DEAE- and CM-cellulose were from Whatman. L-[2,3-$^3$H]Proline (35 Ci/mmol) and L-[U-$^{14}$C]proline (225 mCi/mmol) were purchased from New England Nuclear. L-[5-$^3$H]Proline (29 Ci/mmol) and D-[2-$^3$H]mannose (2 mCi/mmol) were from Amersham/Searle Co. 3-Hydroxyproline was a gift from Dr. Elijah Adams, University of Maryland.

### *Methods*

*Cell Isolation and Culture.* Cloned and mass-cultured amniotic fluid cells were isolated from second trimester amniotic fluid as previously described (Crouch et al., 1978). Cell types were identified on the basis of cellular and clonal morphology, as described by Hoehn et al. (1974). Actively proliferating AF or F clones, containing several hundred cells, were harvested by trypsinization of the entire plate or after isolation of the clone with stainless-steel wells. For subculture, clones were rapidly washed with 0.02% EDTA, dissociated with a 1:1 mixture of EDTA and trypsin (0.25%), and transferred to 60-mm plates. Thereafter, the cells were cultured in DMEM supplemented with 27 mM $NaHCO_3$, 15 mM Hepes, 2 mM glutamine, penicillin (100 units/mL), streptomycin sulfate (100 $\mu$g/mL), and 16% FCS. Cells, near confluence, were passaged to 100-mm plates and subsequently subcultured and split at a ratio of 1:2. Due to the rarity of F cell clones, early passage euploid male F cells were occasionally used after storage in liquid nitrogen. Cells were intermittently screened for mycoplasma by electron microscopy.

*Metabolic Labeling.* Labeling and pulse–chase experiments were performed using just-confluent cultures of AF and F cells (transfer 2–3). For these studies, DMEM was supplemented with sodium ascorbate (50 $\mu$g/mL), $\beta$-APN (80 $\mu$g/mL), and penicillin–streptomycin. Cultures were preincubated for 30 min and labeled for 12–24 h with 20–40 $\mu$Ci/mL [$^3$H]proline or 5 $\mu$Ci/mL [$^{14}$C]proline under conditions producing linear incorporation of isotope in the medium compartment. For pulse–chase experiments, cells were preincubated for 1 h, pulsed with isotope for 30 min, washed three times with DMEM, and chased in medium containing 20 mM L-proline.

To prepare radiolabeled protein, the medium and the first wash of the cell layer were routinely harvested into protease inhibitors [$PhCH_2SO_2F$ (0.2–0.5 mM), MalNet (10 mM), EDTA (0.02 M)] at 4 °C and centrifuged at 400$g$ for 10 min to remove cells. The supernatant was further clarified by centrifugation at 27 000$g$ for 20 min at 4 °C and stored at 4 or −20 °C. The cell layers were washed twice with Tris–saline containing $PhCH_2SO_2F$ (0.2 mM) and stored at −20 °C.

To prepare samples for $NaDodSO_4$-polyacrylamide gel electrophoresis, the cell layers were scraped into 0.5 N $NH_4OH$ containing $PhCH_2SO_2F$ at 0–4 °C. The cells were briefly sonicated, and aliquots of the sonicate were removed for protein determination by the method of Lowry et al. (1951). The proteins of the medium and the cell layer were separately precipitated in the presence of pepstatin A (1 $\mu$g/mL) by the addition of 50% $Cl_3CCOOH$ to a final concentration of 10% and collected by centrifugation at 38 000$g$ for 20 min at 0–4 °C. The pellets were washed with 5% $Cl_3CCOOH$ and dehydrated by successive washes with cold 95% ethanol and absolute diethyl ether. Alternatively, the cell layer was scraped into 0.5 N acetic acid in the presence of pepstatin A and homogenized. The medium (containing pepstatin A) and cell layer were then dialyzed against 0.1 N acetic acid and lyophilized prior to further analysis.

*Preparation of AF Collagens for Structural Analysis.* Cloned AF cells were pooled before the fourth passage and transferred to roller bottles. When the cells were near confluence, the growth medium was supplemented with ascorbate and $\beta$-APN as described above and harvested at daily intervals for up to 5 days. Pooled medium containing protease inhibitors was clarified by centrifugation, and the proteins were precipitated by ammonium sulfate [20% (w/v) at 4 °C]. The pellet was suspended and dialyzed extensively against 0.5 N acetic acid and lyophilized. The protein was dissolved in the same solvent at a concentration of approximately 10 mg/mL and digested with pepsin (1:50, w/w) for 24 h at 4 °C with stirring. Insoluble material was removed by centrifugation, and the supernatant was titrated to pH 8.5 with NaOH to inactivate the pepsin. The material was then dialyzed against Tris–saline. After centrifugation, collagenous proteins were precipitated by the addition of solid NaCl to a concentration of 4.5 M. The precipitate was collected by centrifugation, dialyzed against acetic acid, and lyophilized. Cell-layer collagens were prepared in a similar fashion by homogenization in acetic acid, pepsin digestion (1:10, w/w), and precipitation from 0.5 N acetic acid with 5–10% (w/v) NaCl.

*Salt Fractionation.* Precipitation at neutral pH was performed as described by Chung and Miller (1974) and Trelstad et al. (1976). Collagens in 0.5 M acetic acid (at a concentration of less than 1 mg/mL) were dialyzed briefly against water or dilute acetic acid and then extensively against Tris–saline or 1 M NaCl, 50 mM Tris-HCl (pH 7.5). Lyophilized collagens were occasionally dissolved directly in 1 M NaCl, 50 mM Tris-HCl (pH 7.5). All samples were clarified by centrifuga-

[2] Nomenclature: In accordance with recent usage, $p_C$ and $p_N$ collagen designate molecules with COOH- and $NH_2$-terminal extensions, respectively. Chains intermediate in length between procollagen (pro$\alpha$) and collagen ($\alpha$) chains are termed p$\alpha$ chains. $p_C\alpha$ chains contain intact COOH-terminal extensions and are linked by interchain disulfide bonds. $p_N\alpha$ chains contain only $NH_2$-terminal extensions and, in types I and II collagen, lack interchain disulfide bonds. ProAF2, pAF2, and AF2 designate the pro$\alpha$1(I), $p_N\alpha$1(I), and $\alpha$1 chains of the type I trimer molecule.

tion. Collagens were then fractionally precipitated by the slow addition of solid NaCl [or 5 M NaCl (pH 7.5) for small samples] to concentrations of 1.5, 2.5, 3.5, or 4.5 M. The developing precipitates were stirred overnight at 4 °C, collected by centrifugation (50 000*g*, 45 min, at 4 °C), and washed with the precipitating solution. Each precipitate was then dissolved in 0.5 N acetic acid, dialyzed against acetic acid, and lyophilized. Radioactive samples to be analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis were precipitated in the presence of 50–100 μg of carrier collagen.

*DEAE-cellulose Chromatography.* DEAE-cellulose chromatography was performed as described (Smith et al., 1972; Burke et al., 1977). Whole medium, or redissolved ammonium sulfate precipitates of medium containing inhibitors, was dialyzed against 50 mM Tris-HCl (pH 7.5 at 23 °C), 4 M urea, 2.5 mM EDTA, and 0.2 mM $PhCH_2SO_2F$ at 4 °C. Prior to chromatography, any insoluble material was removed by centrifugation. Samples were applied to a column of DEAE-cellulose (DE-52) (0.8 × 10 cm) and washed with several column volumes. Bound proteins were then eluted using a linear gradient of 0–0.2 M NaCl over a total volume of 400 mL at 40 mL/h. Recoveries were uniformly greater than 70%. Radioactive peaks were then pooled, dialyzed against 1 mM $NH_4HCO_3$, lyophilized, and examined by $NaDodSO_4$-polyacrylamide gel electrophoresis.

*CM-cellulose Chromatography.* Radioactive samples were dissolved in 0.04 M sodium acetate, 6 M urea (pH 4.8). Rat skin collagen (1–5 mg) was added as an internal marker and carrier. Prior to chromatography, samples were denatured by warming to 42 °C for 30 min and clarified by centrifugation at 27 000*g* for 20 min at room temperature. Samples were then applied to 0.8 × 10 cm column of CM-cellulose (CM-52, microgranular), preequilibrated with deaerated buffer at 42 °C, and washed with several column volumes. Bound proteins were eluted using a linear gradient of 0–0.1 M NaCl over a total volume of 200 mL at 40 mL/h. Recovery of radioactivity was in the range of 80–90% for collagen samples and 40–50% for unpepsinized preparations of culture medium.

*$NaDodSO_4$-Polyacrylamide Slab Gel Electrophoresis and Fluorescence Autoradiography.* Proteins were examined by $NaDodSO_4$-polyacrylamide gel electrophoresis on discontinuous methylenebisacrylamide slab gels containing 0.5 M deionized urea (Laemmli, 1970; Studier, 1973), using a 3% stacking gel with a 5, 6, or 10% separating gel. Samples were dissolved in $NaDodSO_4$-urea sample buffer, reduced when indicated with 50 mM DTT, and heated for 2 min at 100 °C. Electrophoresis was performed at room temperature at 30 mA/slab for approximately 3 h. Danyslated or radioactive α chains, β components, and CNBr peptides were included as internal reference standards. After electrophoresis, gel slabs containing radioactive samples were permeated with dimethyl sulfoxide and 2,5-diphenyloxazole for fluorescence autoradiography as described by Bonner and Laskey (1974), dried with heating under vacuum, and exposed at −70 °C to sensitized X-ray film (Laskey and Mills, 1975). Protein was quantitated by scanning the developed films within their linear range with a scanning densitometer (Quick Scan, Helena Laboratories). Gels containing unlabeled proteins were stained with Coomassie blue, and the bands were scanned at 595 nm in their linear range. Interrupted electrophoresis to estimate type III collagen was performed according to Sykes et al. (1977).

*CNBr Digestion and Peptide Mapping.* Radiolabeled collagens and procollagens were characterized by cleavage with cyanogen bromide. Lyophilized samples were dissolved without additional carrier in 0.5–1 mL of 70% formic acid containing 20 mg/mL CNBr. The reaction mixture was briefly flushed with $N_2$, stoppered, and incubated for 5 h at 30 °C with intermittent agitation. Peptides were resolved by $NaDodSO_4$-polyacrylamide gel electrophoresis using a 5% stacking gel and a 12.5% separating gel. For the analysis of CNBr peptides by CM-cellulose chromatography, labeled α chains were mixed with 5–10 mg of carrier α chains, dissolved at less than 10 mg/mL in 70% formic acid, and digested with a fivefold excess (w/w) of CNBr. The lyophilized digest was dissolved in 20 mM sodium formate–40 mM NaCl (pH 3.8) (Lichtenstein et al., 1975) and applied to a column (0.5 × 5 cm) of CM-cellulose equilibrated with the formate buffer at 42 °C and washed with several column volumes. Bound peptides were eluted using a linear gradient of 40–155 mM NaCl over a total volume of 150 mL at 20 mL/h.

*Radioactive Hydroxyproline and Proline Determinations.* Determinations of radioactive hydroxyproline and proline were performed as described by Kruse and Bornstein (1975). For the determination of 3- to 4-hydroxyproline ratios [$^{14}$C]- or [5-$^{3}$H]proline was used as a label, and the ratios in recovered radioactive peaks were determined without further correction.

*Amino Acid Analysis.* Samples were dissolved in constant-boiling HCl, flushed with $N_2$, and hydrolyzed in vacuo at 108 °C for 24 h. Analyses were performed on a Beckman 121 amino acid analyzer modified for single-column microanalysis. Based on the quantitation of 3-hydroxyproline by gravimetric analysis, *Kf* ratios (ninhydrin absorbance divided by concentration) were determined for 4-Hyp/3-Hyp and 4-Hyp/Pro as 1.4 and 0.9, respectively.

*Collagenase Digestion.* Medium or cell-layer proteins were dialyzed against Tris-saline containing 0.2 mM $PhCH_2SO_2F$. Prior to digestion, $CaCl_2$ and MalNEt were added to final concentrations of 5 and 10 mM, respectively. The sample was then incubated for 2 h at 37 °C with 1 μg/mL bacterial collagenase (Advance Biofactures, form III). The reaction was terminated by $Cl_3CCOOH$ precipitation at 0 °C in the presence of pepstatin A (1 μg/mL). Quantitation of collagenous [$^{3}$H]proline-labeled protein was performed by collagenase assay as previously described (Peterkofsky and Diegelmann, 1971; Kruse and Bornstein, 1975).

*Immune Precipitation.* Immune titration of proteins cross-reacting with antibodies to human fibroblast type I procollagen was performed as previously described for fibronectin (Crouch et al., 1978). Backgrounds with control sera were less than 5% of the total radioactivity. The specificity of the precipitation was verified by $NaDodSO_4$-polyacrylamide gel electrophoresis of immune precipitates.

## Results

*Collagen Synthesis and Accumulation in Amniotic Fluid Cell Cultures.* Cloned AF and F cells incorporate radiolabeled proline into collagenous proteins that subsequently accumulate in the culture medium and cell layer. Under conditions that permit linear proline incorporation into medium proteins for at least 24 h, there is a concomitant linear increase in bacterial collagenase-sensitive, hydroxyproline-containing protein in the medium compartment and an accumulation of collagenous protein in the cell layer. Table I shows the distribution of [$^{3}$H]proline-labeled proteins in parallel cultures of cloned AF and F cells after a 24-h labeling period in the presence of ascorbate and β-APN. As indicated by the table, F cells accumulated more labeled protein and significantly more collagen per milligram of cell layer protein that did AF cells. Although we have observed interclonal variation in the absolute levels of incorporation into protein, especially for AF cells, F cells were consistently more active than AF cells in synthesizing

TABLE I: [$^3$H]Proline Incorporation and Collagen Accumulation by AF and F Cells.[a]

| | cpm/mg of protein ($\times 10^{-4}$) | | cpm in collagen/mg of protein ($\times 10^{-4}$)[b] | | % collagenase-sensitive protein | | 3-Hyp/total Hyp | |
|---|---|---|---|---|---|---|---|---|
| | AF | F | AF | F | AF | F | AF | F |
| medium | 62 | 220 | 14 | 141 | 23[c] | 64[d] | 0.12 | 0.03 |
| cells | | | | | | | | |
| acetic acid extract | 145 | 390 | 13 | 130 | 9 | 33 | 0.13 | 0.03 |
| residue | 39 | 76 | 4.7[e]e | 7[e] | | | 0.11 | 0.06 |
| total | 246 | 686 | 32 | 278 | | | | |

[a] Cells were labeled for 24 h with 20 $\mu$Ci/mL of L-[5-$^3$H]proline as described under Experimental Section. [b] Determined by bacterial collagenase digestion. [c] 26 ± 7%, determined with 11 different clones. [d] 62 ± 9%, determined with five different clones. [e] Based on hydroxyproline analyses assuming an equal content of hydroxyproline and proline in collagen.

collagenous proteins and generally released a larger proportion of the newly synthesized collagen into the culture medium.

The proportion of the total radiolabeled protein that is collagenous, as assessed either by collagenase assay (Table I) or by densitometry of collagenase-sensitive bands after resolution of proteins by NaDodSO$_4$-polyacrylamide gel electrophoresis (see below), was characteristic and relatively constant for each cell type under standardized conditions of culture. Approximately one-quarter of the proline incorporated into protein in AF cell culture medium was found in collagenase-sensitive sequences, as compared to nearly two-thirds in F cell culture medium. Forty to fifty percent of the incorporated [$^3$H]proline in the medium of AF cells was in fibronectin, even when the cells were labeled in the presence of ascorbate (Crouch et al., 1978). In contrast, only 15–25% of the incorporated proline label was found in fibronectin in the medium of F cells. As previously described by Priest et al. (1977), a significantly greater proportion of the incorporated proline radioactivity in AF cultures was recovered as 3-hydroxyproline (Table I). Differences between AF and F cells were also consistently observed in the cell layer (Table I).

The [$^3$H]proline-labeled proteins synthesized by AF and F cells were examined by NaDodSO$_4$-polyacrylamide gel electrophoresis and fluorescence autoradiography (Figure 1). The identification and migration of fibronectin on NaDodSO$_4$ gels of AF and F cell medium and cell layer were reported previously (Crouch et al., 1978). The major proteins of the F cell medium migrated similarly to the type I procollagens, p collagens, and $\alpha$ chains of fetal and adult dermal fibroblasts and were sensitive to digestion with bacterial collagenase. The identity of these proteins was confirmed by DEAE- and CM-cellulose chromatography, pepsin digestion, and CNBr peptide mapping (see below). Procollagen intermediates and their derived chains and peptides were identified and characterized by ion-exchange chromatography and two-dimensional electrophoresis as described by Davidson et al. (1975, 1977) for chick type I procollagen. The most abundant collagenous protein found in F medium after short- or long-term labeling is disulfide bonded and comigrated with fibroblast type I procollagen. After reduction, two chains appeared which comigrated with pro$\alpha$1(I) and pro$\alpha$2. In short-term labeling experiments these radioactive chains predominated and were present in a ratio of approximately 2–2.5:1. However, after a 24-h labeling period, components corresponding to p$_C\alpha$1, p$_C\alpha$2, p$_N\alpha$1, p$_N\alpha$2, $\alpha$1, and $\alpha$2 were also prominent (Figure 1). Small amounts of type III procollagen were also present (see below).

In the acetic acid soluble fraction of the F cell layer, the most abundant, collagenase-sensitive chains migrated in the position of $\alpha$1(I) and $\alpha$2 and were present in a ratio of 2:1 (Figure 1). The conversion of procollagen to p collagen and $\alpha$ chains in F cell culture medium and cell layer was demonstrated by pulse-chase experiments (not shown).

The major collagenous proteins of AF medium and cell layer were identified and partially characterized on the basis of their electrophoretic mobility on NaDodSO$_4$-polyacrylamide gel electrophoresis. When proteins accumulated during a 24-h labeling period were examined after reduction with DTT, four major collagenase-sensitive chains were observed (Figure 1). The closely paired chains migrating below fibronectin are derived from a type IV-like procollagen, referred to here as AF1 (Crouch, E., and Bornstein, P., manuscript in preparation). The prominent chain migrating below AF1, designated proAF2, has been found to be derived from a disulfide-bonded procollagen with three identical pro$\alpha$ chains which are structurally and immunologically related to the pro$\alpha$1 chain of type I procollagen (see below). It should be noted, however, that reduced proAF2 chains migrated more slowly than the pro$\alpha$1(I) chains of the F cell medium. The protein migrating below proAF2, in the approximate position of p$_C\alpha$1(I), was found to be structurally related to proAF2. This chain, designated pAF2, is a conversion intermediate derived from proAF2. Since the migration of pAF2 was not influenced by reducing agents, this chain presumably lacks interchain disulfide bonds. In this respect, it corresponds to p$_N\alpha$1(I) and p$_N\alpha$1(II), which contain only NH$_2$-terminal extensions (Davidson et al., 1975; Uitto, 1977). As will be discussed below, variable amounts of types I and III procollagens and intermediates were also present in AF cell cultures but were frequently minor components and were poorly resolved from proAF2 and pAF2 by NaDodSO$_4$-polyacrylamide gel electrophoresis.

*DEAE-cellulose Chromatography of F and AF Cell Medium Proteins.* F and AF cell medium proteins were chromatographed on DEAE-cellulose using conditions similar to those previously described for the isolation and characterization of type I procollagens, p collagens, and procollagen-derived peptides. DEAE-cellulose chromatography of F medium, without prior ammonium sulfate precipitation, gave an elution profile (Figure 2A) similar to that previously described for human and monkey skin fibroblasts (Smith et al., 1972; Lichtenstein et al., 1975; Burke et al., 1977). Peak 1 (Figure 2A, inset) was found to contain mainly components with the mobility of p$_C\alpha$ and $\alpha$ chains on NaDodSO$_4$-polyacrylamide gel electrophoresis. A disulfide-bonded component migrating faster than $\alpha$ chains tentatively identified as the type I procollagen-derived COOH-terminal fragment was also observed. Peak 2 contained a disulfide-bonded protein which migrated in the region of type I procollagen and yielded chains with the mobility of pro$\alpha$1(I) and pro$\alpha$2 after reduction. Smaller

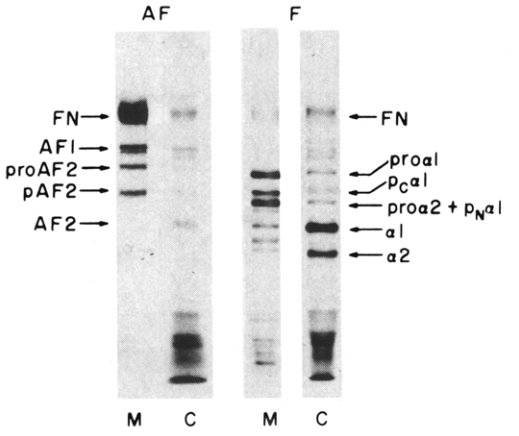
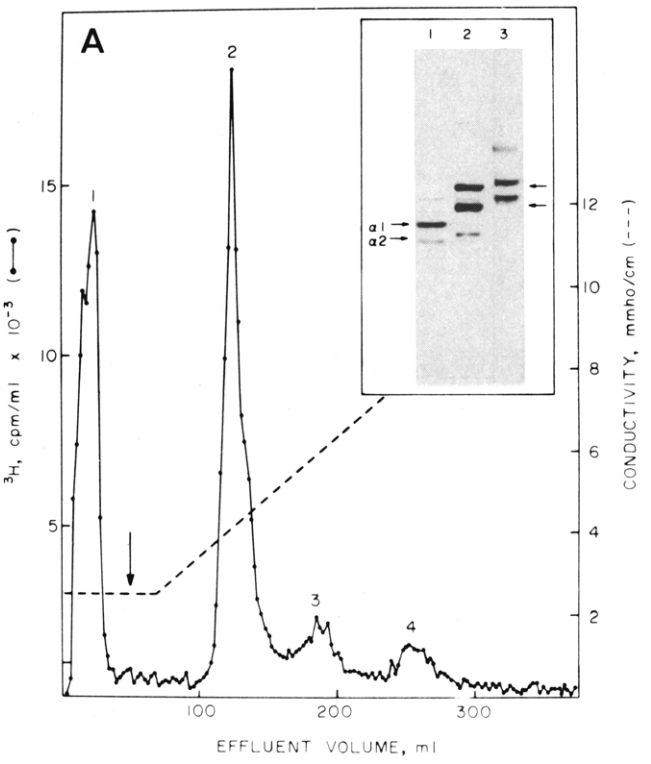



FIGURE 1: Fluorescence autoradiograms of [$^3$H]proline-labeled proteins in AF and F cell culture medium (M) and acetic acid extracts of cell layers (C). Proteins were resolved by NaDodSO$_4$–polyacrylamide gel electrophoresis after reduction with dithiothreitol. Just-confluent cultures were labeled for 24 h with L-[5-$^3$H]proline in the presence of ascorbate and β-APN and processed in the presence of inhibitors as described under Methods. Fibronectin (FN) and collagenous proteins are identified (see text). ProAF2, pAF2, and AF2 designate proα, p$_N$α, and α chains, respectively, of [α1(I)]$_3$. Proα1(III) comigrates with proα1(I). Minor bands in F cell medium below proα2 were identified as p$_C$α2 and p$_N$α2, respectively.

amounts of non-disulfide-bonded chains with the mobility of p$_N$α and α chains were also present. Pepsin digestion of peak 2 material yielded non-disulfide-bonded chains with the mobility of standard α1(I) and α2 chains in a ratio of 2–2.3:1, and a type I peptide pattern was obtained after cleavage with CNBr (not shown). Peak 2 has, therefore, been identified as type I procollagen. Peak 3 contained fibronectin and a disulfide-bonded component or components migrating in the region of procollagen which, after reduction, yielded two chains. These chains were identified as proα1(III) and pα1(III). Peak 4 has been tentatively identified as the proα1(I)-derived NH$_2$-terminal peptide on the basis of its position of elution from DEAE-cellulose (Fessler et al., 1975) and its decreased mobility on NaDodSO$_4$–polyacrylamide gel electrophoresis after reduction, consistent with the presence of intrachain disulfide bonds (Engel et al., 1977).

DEAE-cellulose chromatography of the AF cell culture medium (Figure 2B) gave a distinctly different profile from that obtained with F cell medium. There was no major peak eluting in the position expected for type I procollagen. Instead, several prominent, incompletely resolved peaks eluted in the region occupied by peaks 3 and 4 in the chromatogram of F cell medium. Peak 1 was found to contain AF1 and low-molecular-weight components. Peak 2 contained a variety of non-collagenous and low-molecular-weight proteins, which were not further characterized. When examined by NaDodSO$_4$–polyacrylamide gel electrophoresis, the poorly resolved peaks (4–6) were found to contain mainly fibronectin (Crouch et al., 1978). In addition to fibronectin, peak 5 contained collagenase-sensitive components identified as proAF2 and pAF2 (Figure 2B, inset). The upper component was derived from a protein which migrated in the region of type I procollagen prior to reduction, whereas the lower component migrated in the same position before and after reduction. No bands were observed in the region of p$_N$α2 or α chains. When the proteins recovered in this peak were digested with pepsin, an α chain containing protein was recovered which migrated on NaDodSO$_4$–polyacrylamide gel electrophoresis with an apparent molecular weight of 100 000–105 000, using human type I



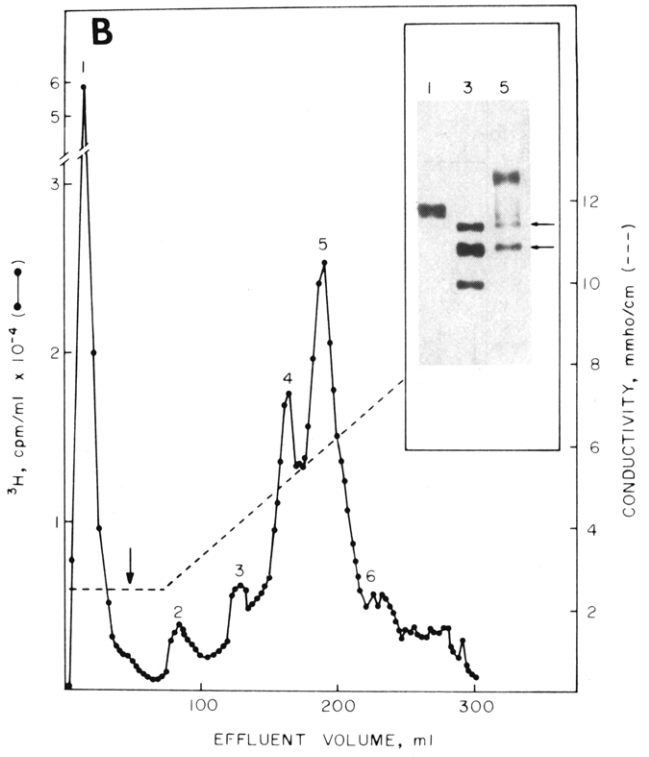




FIGURE 2: DEAE-cellulose chromatograms of [$^3$H]proline-labeled medium proteins of (A) F cells and (B) AF cells. Just-confluent cultures were labeled for 24 h with radiolabeled proline in the presence of ascorbate and β-APN and chromatographed as described under Methods. Arrow indicates start of gradient. Conductivities were measured at 4 °C. Radioactive peaks were analyzed as described in the text. Insets show fluorescence autoradiograms of selected peaks as resolved by NaDodSO$_4$–polyacrylamide gel electrophoresis in the presence of DTT. In A, peak 1 contains type I p$_C$ collagen and collagen, peak 2 contains type I procollagen and p$_N$ collagen, and peak 3 contains type III procollagen and p collagen. In B, peak 1 contains AF1, peak 3 contains type I procollagen, peak 4 contains fibronectin, and peak 5 contains fibronectin and proAF2 and pAF2 (arrows).

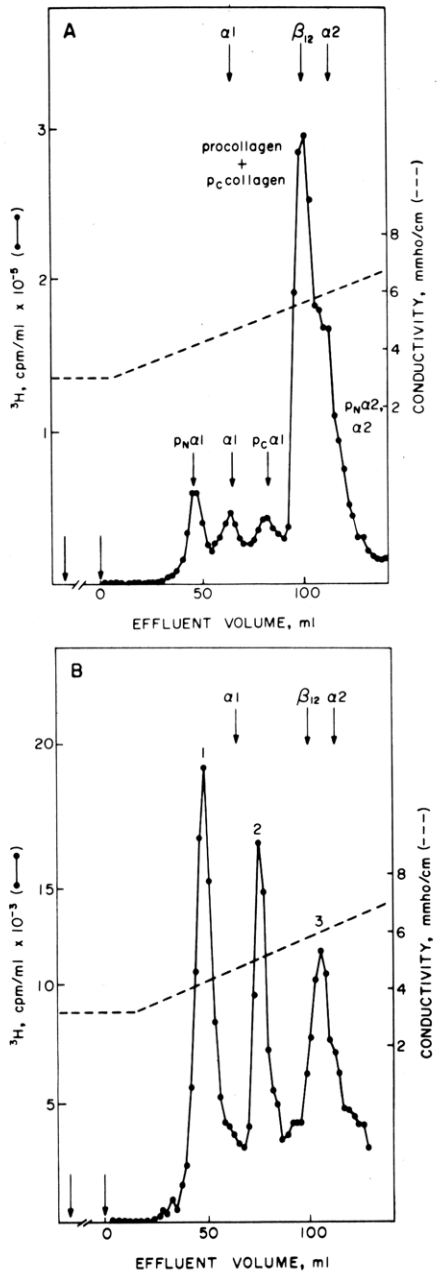



FIGURE 3: CM-cellulose chromatograms of [$^3$H]proline-labeled culture medium proteins of (A) F cells and (B) AF cells. Cells were labeled as described in the legend to Figure 2. Medium, harvested in the presence of inhibitors, was precipitated with $(NH_4)_2SO_4$ [20% w/v) at 4° C] and dissolved in 0.5 N acetic acid containing pepstatin A (5 μg/mL) and dialyzed against CM-cellulose buffer at 4 °C, prior to chromatography in 6 M urea at 42 °C. Arrows indicate the application of the sample and start of the gradient. Conductivities were measured at room temperature. The positions of elution of rat skin collagen carrier chains are indicated. Fibronectin was not recovered from the column. Peaks 1–3 in B contained pAF2 + $p_N\alpha1$, AF2 procollagen, and AF1 + type I procollagen, respectively (see Figure 4 and text).

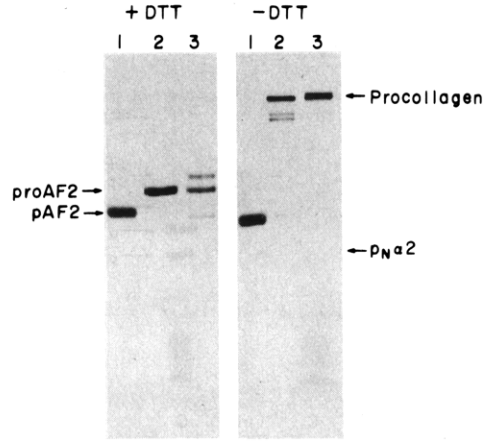



FIGURE 4: Fluorescence autoradiograms of [$^3$H]proline-labeled AF cell medium proteins obtained by chromatography on CM-cellulose (Figure 3B). Proteins were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis (6% acrylamide) before and after reduction with DTT. Numbers refer to peaks in Figure 3B. Peak 1 contains pAF2 and probably some $p_N\alpha1$(I). Peak 2 contains AF2 procollagen. Peak 3 contains AF1, type I procollagen, and $p_N\alpha2$. The chains migrating between procollagen and pro$\alpha$ chains in the absence of DTT presumably represent $p_C$ collagen intermediates.

collagen standards. The pattern of CNBr-produced peptides was similar to that of $\alpha1$(I), except that each of the peptides migrated more slowly than its counterpart from $\alpha1$(I) (see below). Additional peptides, consistent with the presence of some type III procollagen in CNBr digests of peaks 4 and 5, were frequently observed.

Peak 3, when examined before and after pepsin digestion by $NaDodSO_4$–polyacrylamide gel electrophoresis and by CNBr digestion, was found to correspond to type I procollagen. Thus, AF cells produce a small amount of type I procollagen that elutes from DEAE-cellulose in the same position as F cell medium type I procollagen (see Figure 2A). However, this procollagen and its derivative chains and peptides migrate more slowly on $NaDodSO_4$–polyacrylamide gel electrophoresis than the corresponding components from F medium (not shown). Indeed, the components in peak 3 which correspond to pro$\alpha1$(I) and $p_N\alpha1$(I) comigrate on gels with the proAF2 and pAF2 chains eluting in peak 5.

*CM-cellulose Chromatography of F and AF Cell Culture Medium Proteins.* The chromatographic behavior of F and AF cell proteins was also examined by CM-cellulose chromatography under denaturing conditions (6 M urea, 42 °C). This method resolves collagenous components from a variety of low-molecular-weight contaminants, which are not retained by the column, and from fibronectin which appears to bind tightly. When F cell medium proteins were chromatographed without prior reduction, a major peak eluted near the position of $\beta_{12}$ and three smaller peaks eluted earlier in the gradient (Figure 3A). These peaks were identified by $NaDodSO_4$–polyacrylamide gel electrophoresis before and after reduction and by CNBr cleavage. The major peak was found to contain disulfide-bonded components corresponding in mobility to type I procollagen and $p_C$ collagen. The trailing edge of this peak contained chains corresponding to $p_N\alpha2$ and $\alpha2$ chains. The peaks which were resolved earlier in the gradient contained $p_N\alpha$, $\alpha1$, and $p_C\alpha1$ chains.

Again, a distinctively different profile was obtained for the AF cell medium (Figure 3B). Three peaks were eluted within the gradient and migrated near the elution positions of $p_N\alpha1$(I), $p_C\alpha1$(I), and type I procollagen, respectively. However, when the peaks were examined by $NaDodSO_4$–polyacrylamide gel electrophoresis before and after reduction (Figure 4), peak 1 was found to contain nondisulfide-bonded chains comigrating with pAF2, peak 2 contained a disulfide bonded component which migrated with AF2 procollagen prior to reduction and with proAF2 chains after reduction, and peak 3 was found to contain AF1 and variable amounts of type I procollagen. The elution of AF2 procollagen from CM-cellulose at a lower conductivity than type I procollagen is consistent with their relative positions of elution from DEAE-cellulose. The major peaks were further analyzed by CNBr cleavage.

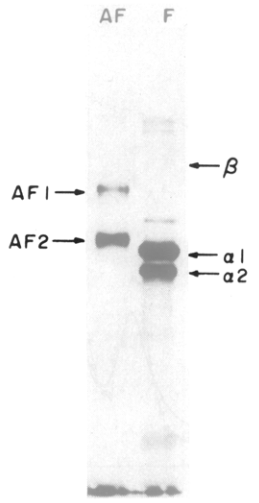



FIGURE 5: Fluorescence autoradiograms of [$^3$H]proline-labeled AF and F cell medium proteins after pepsin digestion. Proteins were resolved by NaDodSO$_4$–polyacrylamide gel electrophoresis (6% acrylamide) after reduction with DTT. Cells were labeled for 24 h as described in Figure 2, dialyzed against 0.5 N acetic acid, digested with pepsin (50 μg/mL, 24 h, 4 °C), and lyophilized after pepsin inactivation. Bands in F cell medium migrating between α chains and the position of β components have been tentatively identified as AF1, αB, and αA. α1(III) is not resolved from α1(I).

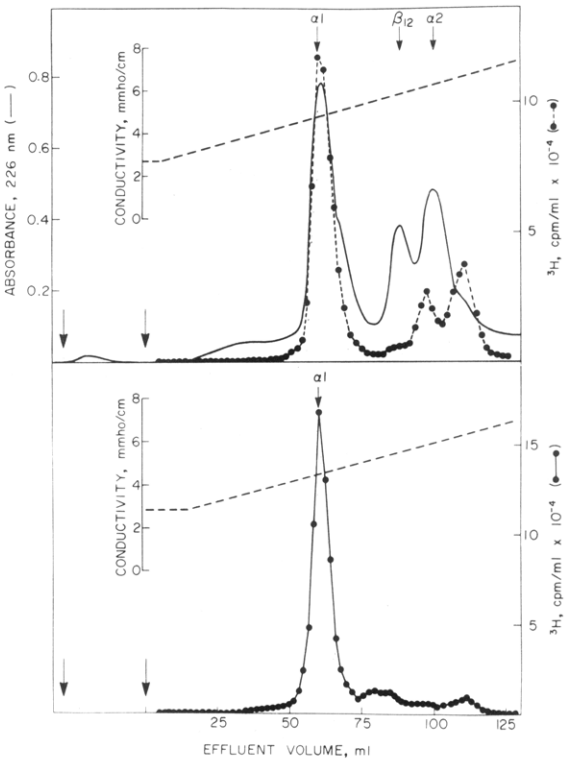



FIGURE 6: CM-cellulose chromatograms of pepsin-digested [$^3$H]proline-labeled F cell (top) and AF cell (bottom) medium proteins after precipitation from 0.5 N acetic acid with 5% NaCl. Radioactive collagens were chromatographed in the presence of carrier rat skin collagen as described under Methods. Peaks were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis. Arrows indicate the application of the sample and start of the gradient. Top: the peaks coeluting with α1 and after α2 contained α1 and α2 chains, respectively, and the peak eluting after β$_{12}$ contained chains identified as α1(III). Bottom: the major peak contained a single chain migrating on NaDodSO$_4$–polyacrylamide gel electrophoresis in the position of AF2.

Peaks 1 and 2 yielded peptide patterns, comparable to those described for purified AF2 (see below), which lacked peptides migrating near the positions of the major α2-derived peptides. In digests of peak 2, the peptide in AF2 corresponding to α1(I)CB6 was also absent, and an increase in radioactivity was observed in the region of α1(I)CB8. This finding is similar to that reported for the CNBr peptides of proα1(I) by Smith et al. (1977) and can be attributed to the presence of an additional nonhelical COOH-terminal sequence in the proα chain, which results in a larger fragment containing α1-CB6 after CNBr digestion.

*Pepsin Digestion of AF and F Proteins.* When unfractionated AF medium or cell layer protein was digested with pepsin and examined by NaDodSO$_4$–polyacrylamide gel electrophoresis, three major pepsin-resistant components were observed (Figure 5). The two upper closely spaced components, which migrated in the region of proα1(I) after reduction, were shown to be derived from AF1 (Crouch, E., and Bornstein, P., manuscript in preparation). The lower component migrated more slowly than α1(I) standards and comigrated with the α chain observed in the AF cell layer after a 24-h labeling period and with the chain isolated by pepsin digestion of DEAE-purified AF2 procollagen. After interrupted electrophoresis of pepsin-digested AF medium, a disulfide-bonded chain with a slightly slower migration than α1(III) standards was resolved from AF2, but this chain accounted for less than 15% of radioactivity in the α-chain region. When F medium or cell-layer protein was similarly examined, two major components were observed which comigrated with α1(I) and α2 (α1/α2, 2–2.5:1) (Figure 5). A small amount of protein with the mobility of type III collagen was also resolved by interrupted NaDodSO$_4$–polyacrylamide gel electrophoresis.

Pepsin digests of AF and F cell proteins were precipitated at acid pH with 5% NaCl (w/v), and the supernatants were examined by NaDodSO$_4$–polyacrylamide gel electrophoresis. Chains which migrated with human αA and αB chain standards were observed. The putative αA and αB chains isolated from the cell layer also eluted from CM-cellulose in positions corresponding to those reported for placental αA and αB (Burgeson et al., 1976; Chung et al., 1976).

The AF2 collagen, obtained by pepsin digestion, could be quantitatively precipitated from 0.5 N acetic acid at 4 °C by the addition of solid NaCl to a final concentration of 5% (w/v). The small amounts of types I and III collagens present were also precipitated by these conditions, leaving type A-B and variable amounts of AF1 in the supernatant. However, when collagens were fractionated by salt precipitation at neutral pH, AF2 remained in the 2.5 M NaCl supernatant and could be subsequently precipitated by the addition of NaCl to a concentration of 3.5–4.5 M.

*CM-cellulose Chromatography of F and AF Cell Pepsin-Digested Medium Proteins.* Three distinct radioactive peaks were observed in CM-cellulose chromatograms of F cell medium collagens, after digestion with pepsin (Figure 6, top). The first peak contained labeled α1 chains which coeluted with carrier α1 chains. CNBr cleavage of these chains, followed by NaDodSO$_4$–polyacrylamide gel electrophoresis, produced a peptide pattern characteristic of α1(I). The peak eluting in the region of β$_{12}$ contained an α chain which was identified as α1(III). The third peak contained a single radioactive chain eluting after α2. The delayed elution of human α2, relative to rat α2, from CM-cellulose has previously been described (Layman et al., 1971). The ratio of α1 to α2 was approximately 2.5:1. A comparable pattern was obtained for F cell layer collagen, except that the type III peak observed in the medium was greatly diminished.

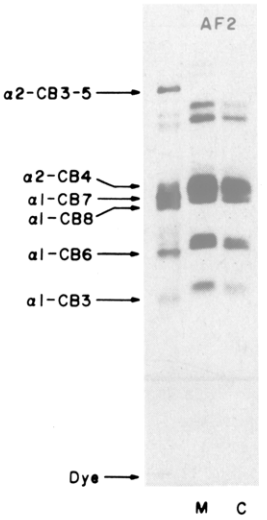



FIGURE 7: $NaDodSO_4$–polyacrylamide gel electrophoresis of CNBr peptides of [$^3$H]proline-labeled F cell medium collagen (first lane) and AF2 chains (second and third lanes). A 12.5% acrylamide separating gel was used. AF2 was obtained by pepsin digestion and CM-cellulose chromatography (Figure 6, bottom). The positions of migration of human skin type I collagen peptides are indicated. The bands above α1-CB6 and below α1-CB3 in F cell collagen are derived from α1(III). Similar patterns were obtained for medium (M) and cell layer (C) AF2 chains.

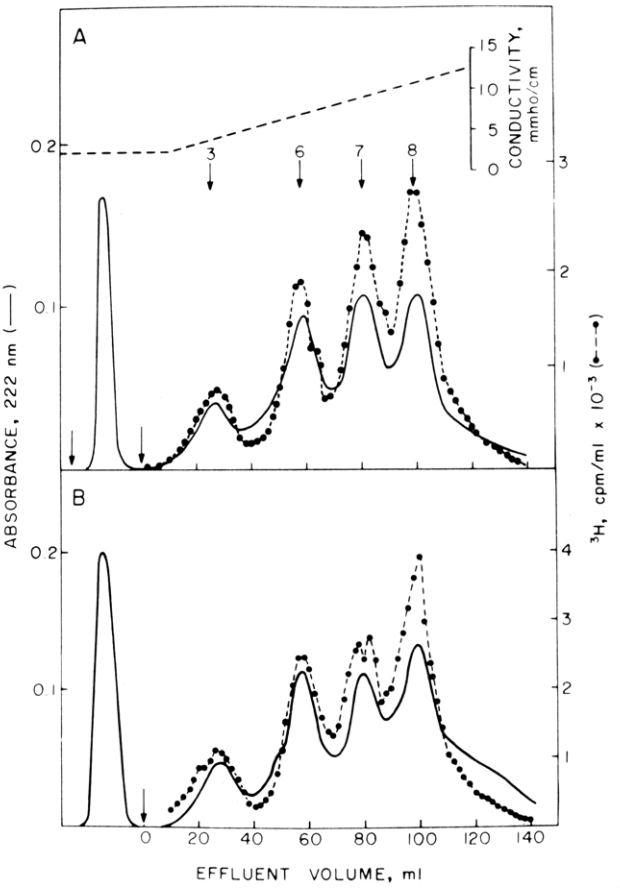



FIGURE 8: CM-cellulose chromatography of CNBr peptides of [$^3$H]-proline-labeled F cell α1(I) (top) and AF2 (bottom). Collagen chains were isolated by CM-cellulose chromatography (Figure 6) and digested with CNBr in the presence of carrier rat skin collagen α1(I) chains. Arrows indicate the application of the sample and start of the gradient. Absorbance and radioactive peaks were identified by $NaDodSO_4$–polyacrylamide gel electrophoresis. Numbers refer to the major CNBr peptides recovered in that peak.

When parallel preparations of AF medium collagen were chromatographed on CM-cellulose, a single major peak of radioactivity was observed in the position of α1(I) (Figure 6, bottom). Essentially all the radioactivity in this peak was localized by $NaDodSO_4$–polyacrylamide gel electrophoresis to a single band migrating slightly more slowly than carrier or F cell α1(I) chains. The small peak eluting immediately after α1 was found to contain residual AF1, and the small peak eluting immediately after α2 contained a radioactive protein migrating slightly more slowly than F cell medium α2. A comparable pattern was obtained with the cell layer. Similar ratios of carrier collagen α1 to α2 were obtained from CM-cellulose, with and without prior pepsin digestion, indicating that selective digestion of the α2 chain had not occurred.

*CNBr Cleavage of AF and F Collagens.* The AF cell radioactive peaks eluting from CM-cellulose in the position of α1(I) (Figure 6, bottom) were cleaved with CNBr and examined by $NaDodSO_4$–polyacrylamide gel electrophoresis. The peptide pattern obtained is shown in Figure 7 and is compared to the CNBr peptides derived from pepsin-treated F cell medium. The F medium peptides comigrated with standard α1(I)-, α2(I)-, and α1(III)-derived CNBr peptides. The pattern of AF2 CNBr peptides is clearly similar to that of α1(I), although as previously noted each AF2-derived peptide migrated more slowly than the corresponding α1(I)-derived F cell medium peptide. When the same peptides were chromatographed on CM-cellulose, all the major radioactive peaks of AF2 coincided with the major absorbance peaks of codigested carrier α1(I) chains (Figure 8, bottom). An essentially identical profile was obtained when radioactive CNBr peptides derived from F cell medium α1(I) chain were similarly chromatographed (Figure 8, top).

*Amino Acid Analyses.* Amino acid compositions of pepsin-digested AF2 and α1(I) human collagen chains are presented in Table II. There is very close agreement between the composition of AF2 and α1(I), with the exception that 3-hydroxyproline, 4-hydroxyproline, and hydroxylysine are all markedly increased in AF2. Based on the expected number of prolyl and lysyl residues in position Y in the Gly-X-Y repeating triplet of the human α1(I) chain (Bornstein and Traub, 1979), the 4-hydroxyproline and hydroxylysine contents of AF2 approach a maximum if hydroxylation is limited to position Y. The composition clearly differentiates AF2 from α1(III), αA, αB, and α1(IV) chains (Bornstein and Traub, 1979). On the basis of the composition alone, it is difficult to clearly differentiate AF2 from type II collagen, but characteristic type II peptides were not observed in CNBr maps (Figures 7 and 8).

*Immune Precipitation.* Additional evidence for the structural relationship of AF2 procollagen to type I procollagen was obtained by immunological methods. An antibody to human type I procollagen was obtained by injection of the purified protein prepared from the culture medium of adult skin fibroblasts. Although the specificity of this antibody is not completely characterized, it completely precipitates fibroblast medium type I procollagen and p collagens but does not precipitate type III or AFl. When dialyzed AF culture medium was titrated with the antiserum, approximately 18% of the nondialyzable radioactivity was precipitated. This percentage agreed well with the percent of total radioactivity in proAF2 + pAF2, as determined by densitometry of fluorescence autoradiograms of culture medium. When immune precipitates of AF medium were examined by $NaDodSO_4$–polyacrylamide gel electrophoresis, the only radioactive bands seen comigrated with proAF2 and pAF2 (data not shown).

*Procollagen Processing.* In addition to the previously described structural relationship of proAF2 to pAF2, a precursor-product relationship of proAF2 to pAF2 was demonstrated by pulse-chase experiments. When AF cells were pulsed for 30 min with [$^3$H]proline and chased for up to 48 h in medium containing an excess of unlabeled proline, a time-dependent shift in radioactivity from the position of migration of proAF2 to pAF2 was observed on NaDodSO$_4$-polyacrylamide gel electrophoresis (data not shown). During the same period, the total radioactivity in proAF2 + pAF2 was essentially constant, and no bands with the mobility of $\alpha$ chains were observed in the medium. However, a time-dependent appearance of pAF2 and AF2 was observed in AF cell layers. These kinetic data suggest that the AF2 chain which appears in the cell layer is generated from cell layer proAF2 and pAF2. We have no evidence for the transfer of collagen from the culture medium to the cell layer under the conditions of these experiments.

## Discussion

Amniotic fluid F and AF cells synthesize, secrete, and accumulate collagenous proteins in cell culture. F cells synthesize predominantly type I with small amounts of type III procollagen. AF cells, however, synthesize considerably less collagen, and the pattern of collagenous proteins, as visualized by NaDodSO$_4$-polyacrylamide gel electrophoresis and ion-exchange chromatography, is distinct. The major proteins in the medium and cell layer of AF cells were found to be a procollagen (proAF2) with three identical pro$\alpha$ chains, structurally and immunologically related to pro$\alpha$1(I), and a type IV-like procollagen, designated AF1 (Figure 1) (Crouch, E., and Bornstein, P., manuscript in preparation). Together these proteins account for 30–40% of the medium radioactivity when cells are labeled for 24 h with [$^3$H]proline in the presence of ascorbate.

AF cells do synthesize variable amounts of another procollagen with two different pro$\alpha$ chains that are present in a ratio of approximately 2:1. Despite a slower migration on NaDodSO$_4$-polyacrylamide gel electrophoresis, relative to F cell type I procollagen, we could identify this protein as type I procollagen. It therefore seems likely that the variable ratio of $\alpha$1 to $\alpha$2 reported by Priest et al. (1977) reflects different relative rates of production or accumulation of type I and AF2 procollagens. AF cells also synthesize small amounts of type III procollagen and collagens with chains tentatively identified as $\alpha$A and $\alpha$B.

AF2 procollagen was distinguished from type I procollagen by the presence of three identical pro$\alpha$ chains, earlier elution from DEAE-cellulose, and later elution from CM-cellulose. In addition, chains and peptides derived from AF2 procollagen were distinguished from their F cell counterparts by their uniformly slower migration on NaDodSO$_4$-polyacrylamide gel electrophoresis. We can identify (proAF2)$_3$ and (AF2)$_3$ as [pro$\alpha$1(I)]$_3$ and [$\alpha$1(I)]$_3$ respectively, because: (a) The amino acid composition of the AF2 $\alpha$ chain closely resembles that of $\alpha$1(I), except for the increased posttranslational modification of prolyl and lysyl residues (Table II). The levels of 3-hydroxyproline, the ratio of total hydroxyproline to proline, and the ratio of hydroxylysine to lysine are greater than previously reported for type I collagen. We have, however, observed interclonal variability in the extent of hydroxylation. (b) AF2 and pAF2 coelute from CM-cellulose with $\alpha$1(I) and p$_N\alpha$1(I), respectively (Figure 3). The different elution of AF2 procollagen from DEAE- and CM-cellulose can be explained on the basis of charge differences between procollagens which contain or lack pro$\alpha$2 chains (see below). (c) The pattern of AF2 CNBr peptides on CM-cellulose or NaDodSO$_4$-polyacrylamide gel electrophoresis is similar to that of $\alpha$1(I)-derived peptides (Figures 7 and 8) and distinct from that of the other known collagen types including II, III, A-B, and IV. The slower migration of AF2-derived peptides on NaDodSO$_4$-polyacrylamide gel electrophoresis is probably secondary to increased posttranslational modification. (d) AF2 procollagen is cross-reactive with antibodies to type I procollagen. (e) The disulfide-bonded collagenase-resistant fragment of AF2 procollagen migrates with that of type I procollagen, and the CNBr pattern of AF2 procollagen is comparable to that of type I procollagen (not shown). (f) AF2 collagen precipitated, at neutral pH, at a NaCl concentration of 3.5 to 4.5 M, as had been shown previously for type I trimer collagen (Chung et al., 1976; Narayanan and Page, 1976).

TABLE II: Amino Acid Composition, in Residues per 1000, of AF2 Compared with $\alpha$1(I).

| amino acid | AF2[a] | $\alpha$1(I)[b] |
|---|---|---|
| 3-Hyp | 15 ± 2 | 0.5 |
| 4-Hyp | 117 ± 3 | 91 |
| Asp | 48 ± 3 | 44 |
| Thr | 18 ± 0.8 | 16 |
| Ser | 36 ± 2 | 36 |
| Glu | 75 ± 1 | 78 |
| Pro | 88 ± 2 | 131 |
| Gly | 329 ± 3 | 331 |
| Ala | 117 ± 3 | 119 |
| Val | 21 ± 2 | 21 |
| Met[c] | 6.9 ± 0.6 | 5.9 |
| Ile | 9 ± 1 | 7 |
| Leu | 21 ± 1 | 19 |
| Tyr | 2.5 ± 0.8 | 2.1 |
| Phe | 13 ± 0.4 | 13 |
| Hyl | 21 ± 2 | 4.2 |
| Lys | 14 ± 1 | 30 |
| His | 3.0 ± 0.5 | 2.1 |
| Arg | 49 ± 2 | 49 |

[a] Values are averages of three determinations (± standard error of the mean) and are uncorrected for hydrolytic losses or for incomplete release of isoleucine. Cysteine and tryptophan were not determined. [b] Analysis for $\alpha$1(I) from fetal human skin determined by Click and Bornstein (1970). [c] Sum of methionine and methionine sulfoxide.

Recently, several laboratories have described a collagen with three identical $\alpha$ chains which appear to be structurally related to the $\alpha$1 chain of type I collagen (type I trimer) (Mayne et al., 1975, 1976; Daniel, 1976; Narayanan and Page, 1976; Little et al., 1977; Benya et al., 1977; Jimenez et al., 1977; Moro and Smith, 1977; Munksgaard et al., 1978). The protein has an amino acid composition and CNBr peptide map that closely resemble those of type I collagen. However, small increases in the extent of posttranslational hydroxylation of prolyl and lysyl residues were reported (Little et al., 1977; Jimenez et al., 1977).

Mayne et al. (1975, 1976) suggested that the synthesis of type I trimer results from altered gene expression due to perturbations of the transcriptional process by agents such as bromodeoxyuridine, factors in embryo extract, or in association with in vitro cellular senescence. Subsequently, type I trimers have been described in association with inflammatory disease (Narayanan and Page, 1976) and with virus-induced neoplasia (Moro and Smith, 1977). With the exception of the TSD4 teratocarcinoma cell-derived protein described by Little et al. (1977), the present study is the only description of a type I trimer procollagen. In addition, AF cells are relatively unique,

since they synthesize higher levels of the trimer as compared to other collagen types, including type I. Little et al. (1977) suggested that the trimer collagen represents an embryonic collagen type, and recent studies have shown that such a collagen is synthesized by normal developing tissues in organ culture (Jimenez et al., 1977; Munksgaard et al., 1978). The finding that fetal AF cells produce significant amounts of type I trimer is, therefore, of particular interest. However, since fetal F cells do not synthesize comparable amounts of trimer and since variable amounts of trimer and type I collagen are synthesized by AF cells, the production of $[pro\alpha1(I)]_3$ presumably reflects differences in the state of differentiation and/or embryologic derivation of AF cells rather than a fixed characteristic of the fetal phenotype. The presence of this molecule in diseased tissues and tumors might reflect a process of "dedifferentiation" and expression of a potential which is lost by certain cell types during tissue development.

The late elution of $[pro\alpha1(I)]_3$ from DEAE-cellulose and its early elution from CM-cellulose suggest that it is a more acidic protein than type I procollagen. Since the chains and major CNBr peptides of AF2 and $\alpha1(I)$ coelute from CM-cellulose, a difference in net charge of the helical domains of AF2 and $\alpha1(I)$ is unlikely. Although the $\alpha1$ chain is more acidic than the $\alpha2$ chain, a number of observations suggest that the ion-exchange properties of type I procollagen and intermediates are determined in part by the relatively negatively charged $NH_2$-terminal extensions (Davidson et al., 1975; Fessler et al., 1975; Smith et al., 1972). As shown in Figure 3, $p_N\alpha1$ is even more acidic than $\alpha1$ chain. Consequently, the ion-exchange chromatographic properties of $[pro\alpha1(I)]_3$ are consistent with its chain composition.

AF collagens demonstrate decreased mobility on Na-DodSO$_4$-polyacrylamide gel electrophoresis, relative to F cell and fibroblast collagens. Our data suggest that the differences responsible for the slower electrophoretic mobility of proAF2 chains are localized to the pepsin-resistant helical domain. Specifically, the disulfide-bonded, collagenase-resistant peptide of $[pro\alpha1(I)]_3$ and the corresponding peptide of type I procollagen migrate similarly, whereas the pepsin-resistant chains continue to demonstrate the differences. These migration differences could result from increased hydroxylation of peptidyl proline and lysine and glycosylation of hydroxylysine throughout the helical domain, since all the large CNBr peptides migrate more slowly than their counterparts in $\alpha1(I)$ (Figure 7). In support of this suggestion, experiments in which AF cells were labeled in the presence of $\alpha,\alpha$-dipyridyl, an iron chelator and inhibitor of prolyl and lysyl hydroxylase, have revealed that the resultant underhydroxylated chains migrate as rapidly as the corresponding chains synthesized by F cells (Crouch, E., and Bornstein, P., manuscript in preparation).

Although we have not yet fully characterized the minor AF collagens with respect to their levels of hydroxylation, the slower migration of AF types I and III collagens suggests that increased hydroxylation and/or glycosylation will be found for these collagens as well. Elevated levels of prolyl hydroxylase, lysyl hydroxylase, and glycosyltransferase activity, which generally decrease with age, have been reported for several human fetal tissues (Anttinen et al., 1973, 1977; Murai et al., 1975; Uitto et al., 1969), and increased hydroxylation of certain fetal collagens has also been described (Barnes et al., 1974; Epstein and Munderloh, 1975; Stoltz et al., 1973). Hydroxylation of a given collagen type can also vary between different tissues of the same organism (Bornstein, 1967). These findings suggest that the increased hydroxylation of the trimer, which far exceeds that previously reported for this protein, may reflect both the fetal origin and embryologic derivation of AF cells. It will, nevertheless, be of interest to determine whether the absence of a pro$\alpha2$ chain or altered kinetics of helix formation secondary to the absence of this chain could contribute to the elevated level of hydroxylation.

AF and F cells differed in the rate and pattern of proteolytic conversion of their respective procollagens to p-collagens and $\alpha$ chains. In pulse-chase and long-term labeling experiments, the F cell procollagen was converted to $p_C$ collagens, $p_N$ collagens, and $\alpha$ chains within several hours in both the medium and the cell layer. In contrast, conversion of medium AF2 procollagen to pAF2 in AF cultures took at least 12 h, and there was little evidence for further conversion of pAF2 to $\alpha$ chains in the culture medium. $\alpha$ chains were, however, observed to accumulate slowly in the cell layer. The slow conversion of $[pro\alpha1(I)]_3$ in vitro is consistent with the isolation of similar collagens from normal embryonic chick tendon and calvaria (Jimenez et al., 1977; Davidson, J. M., and Bornstein, P., in preparation).

In conclusion, euploid fetal epithelioid cells, isolated from human amniotic fluid, synthesize a procollagen with the chain composition $[pro\alpha1(I)]_3$. Pro$\alpha$ chain-specific antibodies, cDNA probes for pro$\alpha$ chain mRNA sequences, and techniques for obtaining viable euploid hybrids of epithelioid and fibroblastic cell types are now available (Rowe et al., 1978; Bryant et al., 1978). Using these techniques it should be possible to determine whether the preferential assembly of $[pro\alpha1(I)]_3$ by AF cells is regulated at the level of transcription or translation or involves primarily posttranslational processes such as chain recognition and association. The assembly of the trimer may be accompanied by increased degradation of pro$\alpha2$ chains if independent regulation of synthesis of this chain does not occur. Indeed, substantial intracellular degradation of newly synthesized procollagen was recently reported (Bienkowski et al., 1978). On the other hand, minor sequence differences could exist between pro$\alpha1(I)$ and chains such as proAF2. There is a precedent for this, since sequence heterogeneity for type II collagen, suggesting the existence of more than one pro$\alpha1(II)$ gene, was recently reported (Butler et al., 1977). Since AF cells synthesize both type I procollagen and $[pro\alpha1(I)]_3$, it will also be important to ascertain whether the relative production of these molecules can be modulated in vitro.

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