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Low Molecular Weight Fibroblast Collagen: Structure, Secretion, and Differential Expression as a Function of Fetal and Cellular Age†

Helene Sage
Department of Biological Structure, University of Washington, Seattle, Washington 98195
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ABSTRACT: A unique low molecular weight collagen that was highly resistant to proteolytic degradation was originally isolated from fetal calf ligamentum nuchae fibroblasts and hence termed FCL-1 [Sage, H., Mecham, R., Johnson, C., & Bornstein, P. (1983) *J. Cell Biol. 97*, 1933–1938]. The differential expression of this protein was studied as a function both of fetal (donor) age and of subcultivation in vitro. Concomitant isolation, subculture, and metabolic radiolabeling experiments performed on cell strains from fetal calf ligament (FCL) and fetal bovine skin (FBS) representing different gestational ages (85–270 days in utero) showed that (a) FCL-1 was synthesized preferentially by fibroblasts from younger animals and (b) expression of FCL-1 diminished as a function of increased passage in culture. Levels of FCL-1, measured as percent of total radiolabeled culture medium protein that precipitated in a concentration range of 20–50% ammonium sulfate, ranged from 22% in FCL 85 cells to 7.7% in FCL 270 (term) cells. FBS fibroblasts at passages 6–10 secreted from 13% to 6% FCL-1, respectively. When cells from an 85-day fetal ligament were allowed to accumulate copious extracellular matrix in vitro, the production of FCL-1 was increased to 32%. FCL-1 was not immunoreactive with polyclonal antibodies directed toward most of the sequences of the interstitial type I and type III procollagens. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the apparent molecular weight of FCL-1 was 13 000 (on the basis of collagen peptide standards) and approximately 30 000 (on the basis of globular protein standards). Incubation with bacterial collagenase produced a stable cleavage product of M_r 8000 (by collagen standards) or 17 000 (by globular standards). In contrast, pepsin removed a small peptide of approximately 1000–2000 in molecular weight from FCL-1, and a gradual but progressive proteolysis of the collagen was observed over a period of 1–6 h. Pulse–chase studies revealed a secretion time of approximately 60 min for FCL-1, without the appearance of any processed, intermediate forms. These studies confirm that FCL-1 represents a novel member of the collagen gene family that manifests differential expression as a function of development.

The collagen gene family encompasses 10 distinct types that, collectively, include a minimum of 18 structurally unique α chains (Bornstein & Sage, 1980; Miller, 1985). In view of recent data confirming several unusual variations in the primary structure of the collagen triple helix, Miller (1985) has proposed a new classification scheme for these proteins based on three groups: group I collagens have a continuous triple helix with α chains of $M_r \geq 95\,000$; group II collagens, also composed of polypeptide chains of $M_r \geq 95\,000$, have triple helical regions interrupted by nontriple helical sequences; group III collagens contain triple-helical segments with chain lengths of $M_r < 95\,000$. Studies on the structure of collagen genes have also indicated several different groups on the basis of nucleotide sequence homology, codon usage, conservation of exon size, frequency of introns, and the total size of the gene [for a review, see Boedtker et al. (1983)]. Among the vertebrate collagens, Lozano et al. (1985) have shown that type IX collagen belongs to a novel class of collagen genes that is distinct from the class of genes encoding the fibrillar collagen types I–III.

Type I collagen, the most abundant protein in connective tissue, is essential for the later stages of normal embryonic development (Löhler et al., 1984). This requirement is due, at least in part, to the specific interactions between mesenchymal cells and their extracellular matrix (ECM),[1] of which type I and other collagens form a substantial proportion. The fetal calf ligamentum nuchae is an example of a developmental system in which the component fibroblastic mesenchymal cells

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[1] Abbreviations: BSA, bovine serum albumin; FCL, fetal calf ligament; FBS, fetal bovine skin; DMEM, Dulbecco–Vogt-modified Eagle's medium; DEAE, diethylaminoethyl; CM, carboxymethyl; DTT, dithiothreitol; ECM, extracellular matrix; β-APN, β-aminopropionitrile fumarate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris–saline, 0.15 M NaCl and 50 mM Tris-HCl, pH 7.5; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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undergo differentiation as a consequence of inductive, "homotypic" interaction with their secreted ECM (Mecham et al., 1984a). In an earlier study, fetal calf ligament (FCL) fibroblasts from animals of later gestational age synthesized significantly more elastin, a major component of the ligament ECM, than did cells from the first or second trimester (Mecham et al., 1981). Moreover, the phenotypic expression of this protein was stabilized by the ligament ECM in vitro (Mecham et al., 1981, 1984a). Confirmatory studies on developing sheep nuchal ligament showed an increase in elastin mRNA, and a concomitant decrease in type I procollagen mRNA, just before birth (Davidson et al., 1982).

Examination of the differential expression of collagen genes in FCL fibroblasts led to the discovery of an apparently unique, low molecular weight collagen termed FCL-1 (Sage et al., 1983a). This collagen displayed several unusual structural features, including a marked resistance to proteolytic degradation and a molecular weight of approximately 13 000 (by collagen peptide standards). In further structural studies, we now show that neither [^{3}H]mannose nor [^{3}H]glucosamine was incorporated into the polypeptide chain(s) of FCL-1 and that the protein itself was resistant to chondroitinase ABC and to neuraminidase. Molecular sieve chromatography under native conditions indicated that FCL-1 was not secreted as a polymer of higher molecular weight. FCL-1 contained a collagenase-resistant peptide of M_r 8000 and a pepsin-sensitive domain of M_r 1000–2000. Pulse–chase biosynthetic analysis indicated that the initial secretion product appeared in the culture medium within 1–2 h and had an apparent molecular weight of 13 000. Moreover, this unusual collagen was found to be secreted by fetal calf fibroblasts from both ligamentum nuchae and skin, preferentially by cells at lower passage numbers. FCL cells cultured from tissues at earlier stages of fetal development synthesized proportionally more FCL-1 than those from second or third trimester animals. As a potentially novel member of the collagen gene family, FCL-1 fulfills certain of the criteria for inclusion within this class of extracellular (matrix), triple helical, aggregate-forming molecules (Ninomiya et al., 1984).

Experimental Procedures

Cell Culture. The cells used in this study were provided by Dr. Robert Mecham (Department of Medicine, Jewish Hospital at Washington University Medical Center, St. Louis, MO). Fibroblasts were isolated from the ligamentum nuchae (FCL) and skin (FBS) of fetal calves, as described by Mecham et al. (1981). Explant cultures from fetuses of different gestational ages, ranging from 85 to 270 days (term), were grown in DMEM (Gibco Laboratories, Grand Island, NY) containing antibiotics and 10% by volume fetal bovine serum [all experiments were conducted with one lot of serum, no. 100424, Sterile Systems (Hyclone), Logan, UT] and were subcultivated as previously described (Sage et al., 1983a).

Metabolic Labeling. Nearly confluent cultures at different passages were incubated with [^{3}H]proline in serum-free DMEM containing 50 μg/mL sodium ascorbate and 64 μg/mL β-APN according to Sage & Bornstein (1982). Experimental conditions have been detailed in the respective table and figure legends. L-[2,3-^{3}H]Proline (33 mCi/mL; lot no. 13) and L-[2,3,4,5-^{3}H]proline (102 Ci/mmol; 3.77 TBq/mmol) were purchased from Amersham/Searle Co., Arlington Heights, IL. D-[2-^{3}H]Mannose (13.4 Ci/mmol; Amersham, lot no. 39) and D-[6-^{3}H]glucosamine hydrochloride (30.3 Ci/mmol; Amersham, lot no. 41) were used in conjunction with DMEM supplemented with 50 mg/L glucose, 1.1 g/L sodium pyruvate, 50 μg/mL sodium ascorbate, and 64 μg/mL β-APN. radiolabeling experiments were routinely performed in 150-mm dishes in 8–10 mL of medium per dish. Cells were trypsinized and counted by a hemocytometer both before and after incubation with isotope.

Purification of FCL-1. Radioactive culture medium proteins were fractionated by ammonium sulfate precipitation in the concentration ranges 0–20% and 20–50% (weight to volume ratio), respectively, in the presence of phenylmethanesulfonyl fluoride, *N*-ethylmaleimide, and ethylenediaminetetraacetic acid (Sage & Bornstein, 1982). Ion-exchange chromatography on DEAE-cellulose and CM-cellulose in 6 M urea was performed as previously described by Sage et al. (1983a) and Sage & Bornstein (1982). Molecular sieve chromatography on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ) was performed at room temperature in a Tris–saline buffer. Peptide standards were CNBr-derived fragments from rat skin type I collagen that were initially purified by CM-cellulose and/or phosphocellulose chromatography.

Protease Digestion. An aliquot of 46 μg (0.13 unit) of chondroitinase ABC (provided by Dr. Thomas Wight, Department of Pathology, University of Washington, Seattle, WA) was incubated with 25 000 cpm of [^{3}H]Pro-FCL-1 in a total volume of 53 μL of 0.3 M Tris, 0.6 mg/mL BSA, and 1.8 mM sodium acetate buffer, pH 8.0, for 3 h at 37 °C. A total of 1 milliunit of *Vibrio cholerae* neuraminidase (Calbiochem-Behring, La Jolla, CA, lot no. 010816) was incubated with 21 000 cpm of [^{3}H]Pro-FCL-1 in 30 μL of 0.2 M sodium acetate buffer containing 5 mM calcium acetate, pH 5.5, at 37 °C for 19 h under nitrogen (Fukuda & Hakomori, 1979). The reactions were terminated by addition of an equal volume of Laemmli sample buffer containing 50 mM DTT, and the digests were analyzed by SDS–PAGE. Aliquots incubated without enzyme served as controls. In addition, control digests were performed on [^{3}H]glucosamine and [^{3}H]proline-labeled culture medium proteins isolated from bovine aortic endothelial cell culture medium.

Proteolytic studies with trypsin (Worthington Biochemical Corp., Freehold, NJ; TPCK, 142 units/mg), bacterial collagenase (Advance Biofactures, Lynbrook, NY; form III, 2700 units/mg), and pepsin (Worthington Biochemical Corp.; 2 times crystallized, 2828 units/mg) were conducted as previously described (Sage & Bornstein, 1982; Sage et al., 1983a). Further details of experimental conditions have been included in the figure legends.

Pulse–Chase Studies. (*a*) *Culture Media.* One 150-mm dish of FCL 85 cells was used for each of five time points. The cells were preincubated for 15 min in DMEM containing ascorbate and β-APN as described and were subsequently pulsed for 1 h with 800 μCi of L-[2,3,4,5-^{3}H]proline. One dish was retained in the presence of isotope for 15 h (continuous label). The pulse media were removed from the other four dishes; the cells were washed and subsequently incubated with chase media (DMEM containing ascorbate, β-APN, and 20 mM proline) for 2, 4, 8, and 15 h, respectively. Culture medium proteins were precipitated at ammonium sulfate concentrations of 0–20% and 20–50% and were collected by centrifugation at 20000*g* for 30 min. Pellets were dissolved in a 6 M urea–50 mM Tris-HCl buffer, pH 8.0, containing protease inhibitors, prior to dialysis against 0.1 M acetic acid at 4 °C and lyophilization. Each sample (two ammonium sulfate fractions per time point) was divided into two equal aliquots: One was dissolved in Laemmli buffer and subjected in toto to SDS–PAGE, while the other was incubated with 0.5 μg of bacterial collagenase for 3 h at 37 °C (total reaction volume of 25 μL in Tris–saline containing 66 mM calcium

acetate, pH 7.4), prior to SDS–PAGE.

(*b*) *Cell Layers.* One 150-mm dish of FCL 85 cells was used for each time point. The cells were pulsed for 10 min as described for the culture medium collection. One dish was continuously labeled for 15 h; the others were washed twice and chased as described above for 0, 10, 20, and 40 min. The cell layers were washed briefly with DMEM containing protease inhibitors and were subsequently scraped into 5 mL of 1 M NaCl–50 mM Tris-HCl buffer, pH 7.5, containing 0.2 mM ethylenediaminetetraacetic acid and 2 mM phenylmethanesulfonyl fluoride. The cellular material was extracted overnight by stirring at 4 °C and was subsequently centrifuged at 20000*g* for 1 h. The insoluble material was washed several times with H_2O and was analyzed by SDS–PAGE. Protein was precipitated from the supernatants by the addition of 100% trichloroacetic acid to a final volume of 10% in the presence of pepstatin (10 μg) (Peninsula Laboratories, Inc., Belmont, CA). The pellets were resuspended in Tris–saline containing 50 mM calcium chloride, and equal aliquots were incubated with bacterial collagenase or without enzyme (Sage & Bornstein, 1982). The controls and collagenase digests were analyzed by SDS–PAGE.

Radioimmunoprecipitation and Immunoblotting. Radioimmunoprecipitation of [^{3}H]proline-labeled fibroblast culture medium protein was performed as previously described with the following rabbit antibodies (a gift from Paul Bornstein, University of Washington, Seattle, WA): anti-rat type I procollagen, anti-human type I collagen, anti-bovine type III procollagen, and anti-bovine 43K protein (Sage et al., 1984, 1983b). All antisera were IgG fractions, and the latter two were in addition affinity-purified against bovine type III procollagen and 43K protein, respectively. Immune complexes were precipitated with sheep anti-rabbit IgG, and normal rabbit IgG was used as a specificity control (both purchased from Miles Laboratories, Inc., Elkhart, IN). The radioimmunoprecipitates were analyzed by SDS–PAGE under reducing conditions and were quantitated by liquid scintillation counting.

Immunoblotting was performed according to the method of Towbin et al. (1979), as described further by Crawford et al. (1985). Samples were electrophoretically transferred to nitrocellulose and incubated with the antibodies described above. Positive specificity control samples included bovine type I and type III procollagen, type I collagen, a CNBr digest of rat skin type I collagen, and 43K protein. ^{125}I-Labeled *Staphylococcal* protein A (8 Ci/μg, 95 μCi/mL) was purchased from New England Nuclear.

Other Procedures. SDS–PAGE was performed according to Laemmli (1970) in the presence of 0.5 M urea. Protein standards were purchased from Bethesda Research Laboratories (Bethesda, MD) and from Pharmacia Fine Chemicals (Piscataway, NJ). Autoradiography was performed with EN^{3}HANCE according to the manufacturer's instructions (New England Nuclear, Boston, MA), and protein staining with Coomassie brilliant blue was as described (Sage & Bornstein, 1982). Quantitation by scanning densitometry was performed on a Quick Scan (Helena Laboratories, Beaumont, TX) or a GS300 transmittance–reflectance scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA) in conjunction with a computer-assisted morphometric package. These programs analyze serially sectioned objects by digitizing contours and computing areas and perimeters.

Results

Four types of fetal bovine fibroblasts were examined in this study: (1) FCL (ligament) cells from early gestation (85 and 95 days), (2) FCL cells from midgestation (174 days), (3) FCL cells from late gestation (250 and 270 days), and (4) FBS (skin) cells from late gestation (270 days). In addition to measuring the production of FCL-1 by these cells as a function of donor age, we also investigated the relationship of FCL-1 biosynthesis to two parameters encountered during cell growth in vitro: (1) passage number and (2) accumulation of an extensive ECM.

FCL 85 ("young") cells[2] were generally smaller and less elongated than FCL 270 ("old") cells. In addition, they exhibited shorter doubling times (18–24 h) and higher postconfluent densities, compared to the older FCL cells (Mecham et al., 1984a). However, there was variability with respect to these properties among several isolates of cells from fetuses of similar ages which was at least partially due to tenure in vitro. For that reason we have made comparisons among several strains of both young and old cells of the same passage which were subcultivated and radiolabeled according to identical protocols.

Qualitative Analysis of Protein Secretion by FCL Fibroblasts. A comparison of the secretory phenotypes of FCL 85 and FCL 270 cells is shown by DEAE-cellulose chromatography of [^{3}H]proline-labeled culture medium proteins that were initially precipitated by ammonium sulfate in a concentration range of 20–50% (Sage et al., 1983a) (Figure 1). The distribution of cpm in peaks I and IV was significantly different between the two cell strains. The peaks designated by Roman numerals were pooled and analyzed by SDS–PAGE, as shown in Figure 2 for the FCL 85 cells. Proteins that did not bind to the DEAE-cellulose are shown in lane I (equivalent to fraction I, Figure 1, top panel). With the exception of type I collagen, small amounts of type III collagen, and possibly tropoelastin, most of these components remain unidentified. Lane II contained type VI collagen (unlabeled arrow, M_r 140000) (Crawford et al., 1985). Peak III (Figure 1) was primarily composed of type I procollagen (not shown). FCL-1 was eluted at the end of the gradient, at approximately 160 mM NaCl (peak fraction IV, Figure 1, and lane IV, Figure 2). A noncollagenous protein of M_r 43000 (43K) copurified with FCL-1 (lane IV) (Sage et al., 1984, 1983a). Both FCL-1 and the 43K protein were readily detected by staining with Coomassie blue (lanes IV-C, ±DTT). The M_r 68000, nonradioactive band in lane IV-C was BSA, which binds to the 43K protein (Sage et al., 1984). The molecular weight of FCL-1 was approximately 30000 by globular reference protein standards and 13000 by collagen peptide standards (Sage et al., 1984). Although interchain disulfide bonds were absent, the slight shift to a lower mobility on SDS–PAGE gels after reduction suggested the presence of intrachain disulfide bonding (Figure 2, compare lanes IV ± DTT). SDS–PAGE analysis of the FCL 270 DEAE-cellulose fractions (Figure 1, bottom panel) showed qualitatively the same proteins but a significantly decreased proportion of FCL-1 (not shown; quantitation presented in Table I).

Quantitative Changes in FCL-1 Production as a Function of Donor Age and of Subculture. Quantitative analysis of the synthesis of FCL-1 by FCL fibroblasts as a function of fetal age was performed on FCL 95 and FCL 250 cells, both at the fourth passage. These two strains were cultivated concomitantly and were controlled experimentally as closely as possible. The younger cells secreted higher levels of FCL-1, compared

[2] Fetal calf ligament and fetal bovine skin fibroblast strains are identified by the abbreviation FCL and FBS, respectively, followed by the age of the fetus from which the cells were derived. For example, FCL 85 denotes a cell strain from the ligament of an 85-day fetus.

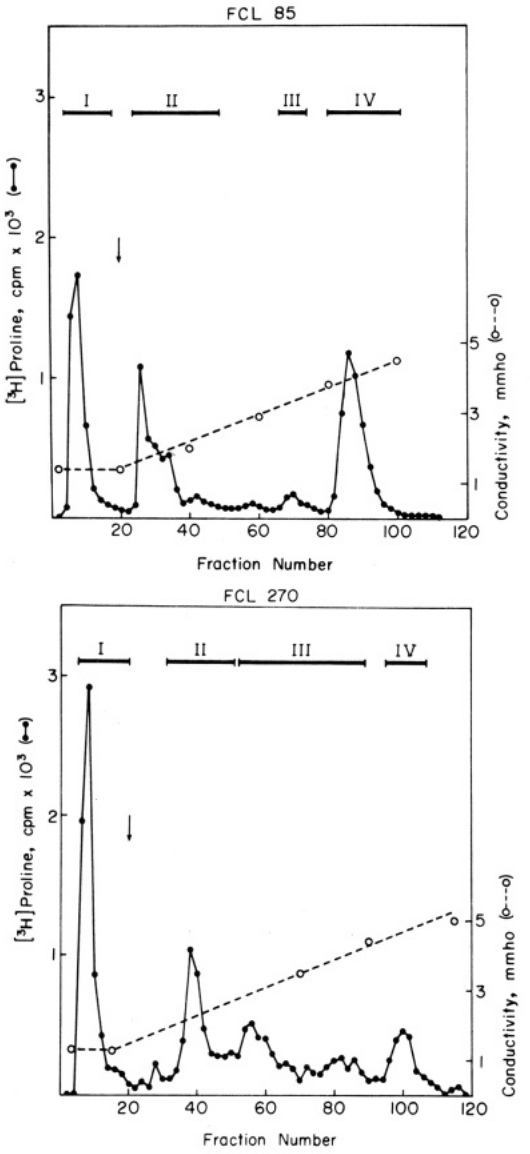

FIGURE 1: Chromatographic analysis of culture medium protein secreted by FCL fibroblasts isolated from donors of early and late gestational age. Cells were cultivated under identical conditions and were incubated just prior to confluence for 18 h with [2,3-^{3}H]proline in serum-free DMEM supplemented with ascorbate and β-APN, as described under Experimental Procedures. The culture medium proteins that were precipitated from a 20 to 50% ammonium sulfate concentration were subsequently fractionated on DEAE-cellulose at 4 °C. Arrows indicate inception of gradient elution (0–200 mM NaCl), and Roman numerals indicate pooled fractions. (Top panel) FCL 85 fibroblasts (passage 6); (bottom panel) FCL 270 fibroblasts (passage 4). Each elution pattern represents 2.05×10^7 cells.

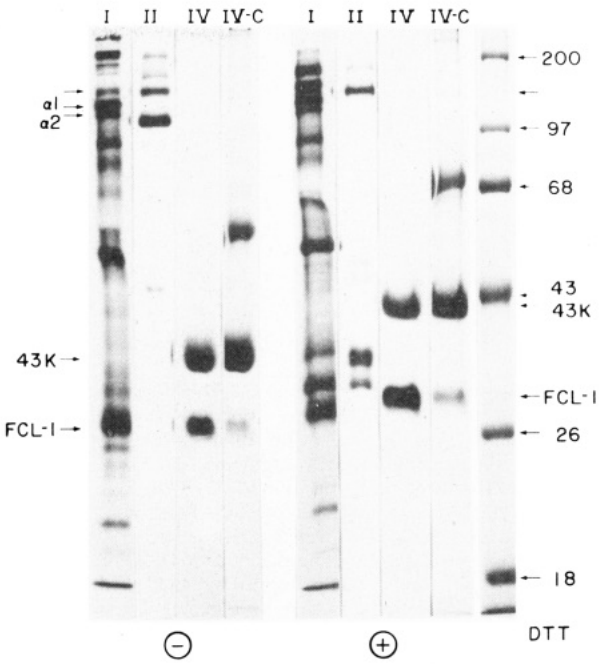

FIGURE 2: SDS–PAGE analysis of proteins secreted by FCL 85 fibroblasts. Culture medium proteins were biosynthetically radiolabeled and fractionated by ion-exchange chromatography as described in Figure 1. Pooled fractions were analyzed on 5%/10% composite SDS gels in the presence and absence of DTT. Proteins were visualized by fluorescence autoradiography (lanes I, II, and IV) or by staining with Coomassie blue (lanes IV-C). Roman numerals correspond to chromatographic fractions shown the top panel of Figure 1. Lane I contained 0.52% of the total cpm in the respective DEAE-cellulose fraction, lane II contained 0.14% of the total cpm in fraction II, and lane IV contained 0.75% of the total cpm in fraction IV. The exposures for each lane have accordingly been adjusted to represent equal cpm. The 43K protein and FCL-1 have been identified in lanes IV and IV-C, ±DTT. The α1 and α2 chains of type I collagen are shown in lane I, ±DTT, and type VI collagen is indicated by the unlabeled arrow (lane II, ±DTT). The molecular weights of globular protein standards have been included on the far right of the figure.

to the older cells, when calculated as percent of the 20–25% ammonium sulfate precipitate (18.8% vs. 7.5%) and as percent of total [^{3}H]Pro-labeled culture medium protein (4.4% vs. 2.4%), respectively. When several other pairs of young and old cells were compared in this manner, similar results were reproducibly obtained (data not shown).

Since it was apparent from earlier studies that the degree of subcultivation was an important variable in assessing levels of protein synthesis by cells in vitro, we examined FBS 270 fibroblasts at passages 6, 8, and 10 by DEAE-cellulose chromatography (data not presented). In Table I are shown the relative amounts of FCL-1 secreted into the culture medium by FBS 270 cells at the different passages. While FCL-1 represented 13% of the total protein in the 20–50% ammonium sulfate precipitate in both sixth and eighth passage cells, it comprised only 6% in the tenth passage cells. When expressed as percent of total protein synthesis, the proportionality in the decrease of FCL-1 production in the late passage cells was maintained (see footnote *c* of Table I). FCL 174 cells were also measured for FCL-1 production at passages 6 and 11. As shown in Table I, there was a slight decrease in FCL-1, as percent of total protein in the 20–50% ammonium sulfate precipitate, in the later passage cells (13.5% vs. 16%), which was accompanied by a general decrease in the incorporation of radiolabel into secreted protein (1.0×10^5 vs. 7.7×10^5 cpm in FCL-1). This general decrease in protein synthesis and radioisotope incorporation is a consequence of cellular aging in vitro. Mecham et al. (1984a) have observed a similar effect with respect to elastin production by FCL cells.

From Table I a comparison can also be made among FCL 85, FCL 174, and FCL 270 fibroblasts of similar passage numbers regarding FCL-1 production. This collagen comprised 22% of FCL 85 protein (20–50% ammonium sulfate fraction), 16% of FCL 174 protein, and 7.7% of FCL 270 protein. These results confirm those shown in Figure 1 (for FCL 85 and 270 cells at passage 4) and those obtained for FCL 95 and 250 cells at passage 4. As the age of the fetal donor increased, the production of FCL-1 relative to total secreted protein decreased.

In a related experiment, FCL 85 cells were permitted to overgrow for 3.5 weeks, with the subsequent production of an extensive ECM. They were found to produce the highest levels of FCL-1, relative to total protein in the 20–50% ammonium

Table I: Synthesis of FCL-1 by FCL and FBS Fibroblasts as a Function of Subcultivation[a]

cell strain	passage no.	cpm of [^{3}H]Pro-FCL-1[b] × 10^5	FCL-1 (% of protein in 20–50% precipitate)[c]
FCL 85	6	8.84	22.2
FCL 85–ECM[d]	5		32.0
FCL 174	6	7.72	16.3
	11	1.02	13.5
FCL 270	4	3.24	7.7
FBS 270	6	10.0	13.5
	8	8.19	13.4
	10	0.66	5.8

[a] Cells were cultivated and labeled under identical conditions with the same respective lots of reagents; all experiments were conducted over a 3-month period. FCL and FBS fibroblast strains were grown in DMEM containing 10% fetal calf serum and were passaged when the cells reached confluence at a split ratio of 1:3. Cells were incubated in serum-free DMEM containing ascorbate, β-APN, and [2,3-^{3}H]proline (20 μCi/mL) for 18 h. [b] FCL-1 was purified from a 20–50% ammonium sulfate precipitate of culture medium by DEAE-cellulose chromatography. Quantitation was performed by liquid scintillation counting of effluent column peaks and by scanning densitometry after SDS–PAGE of pooled column fractions. Reproducibility of recovery from ion-exchange chromatography was within 5% for all samples. The cpm values in FCL-1 have been normalized to equal numbers of cells for each of the strains and represent 10 150-mm dishes (22.7 × 10^6 cells) per sample. [c] [^{3}H]Pro-FCL-1, expressed as percent of total radiolabeled protein secreted into the culture medium, recovered in the 20–50% ammonium sulfate precipitate. Levels of [^{3}H]proline-labeled protein in the 0–20% ammonium sulfate precipitate were consistently proportional to those in the 20–50% precipitate (±2%). [d] One 150-mm dish of FCL 85 cells was allowed to overgrow with concomitant synthesis of a copious extracellular matrix (ECM) for 3.5 weeks; culture medium was changed twice weekly. Cells were incubated as described in footnote *a*, except for a labeling time of 36 h with [2,3,4,5-^{3}H]proline at 25 μCi/mL. [^{3}H]Pro-FCL-1, as percent of total protein in the 20–50% ammonium sulfate precipitate, was measured by scanning densitometry after SDS–PAGE of the entire precipitate. FCL-1 comprised 12.9% of the total ammonium sulfate precipitate (0–20% plus 20–50% fractions).

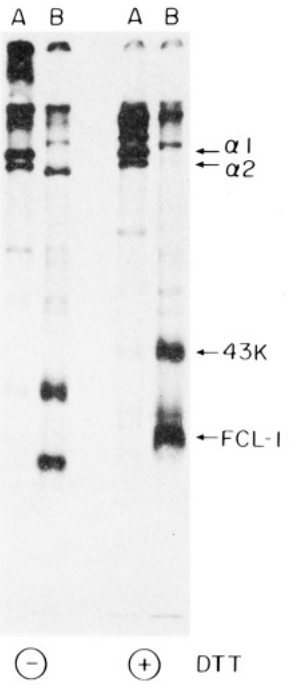

FIGURE 3: Synthesis of FCL-1 by FCL 85 fibroblasts which have accumulated an extensive extracellular matrix in vitro. FCL 85 cells were grown and radiolabeled as described in Table I (see footnote *d*). Culture medium proteins were precipitated at concentrations of 0–20% (lane A) and 20–50% (lane B) ammonium sulfate and were analyzed on a 5%/10% composite SDS gel in the presence and absence of DTT. The fluorescence autoradiograph shows FCL-1 and 43K proteins (B), as well as the α1 and α2 chains of type I collagen (A). To each lane A was applied 1.4 × 10^5 cpm, representing 16.7% of the total recovered cpm in this fraction. To each lane B was applied 1.9 × 10^5 cpm, representing 33.3% of the total recovered cpm in this fraction.

sulfate fraction (32%) (Table I, FCL 85–ECM) or in the 0–20% plus 20–50% fractions (13%), of any of the fibroblast strains that we investigated. The absolute levels of incorporated radioactivity could not be compared to the other cells, however, due to differences in the labeling time and specific activity of the isotope used (see footnote *d* of Table I). SDS–PAGE of the 0–20% and 20–50% ammonium sulfate fractions of FCL 85–ECM culture medium protein is shown in Figure 3. It can be seen that FCL-1 comprises a major portion of the proteins in lane B. At present we feel that most of the FCL-1 produced in vitro is secreted into the culture medium. Experiments in which cell layers were extracted and analyzed for collagenase-sensitive components did not indicate that FCL-1 was associated either with the cell surface or the ECM (see pulse–chase section). In addition, extraction of FCL cells grown on ligament substrata failed to reveal FCL-1 associated with this matrix.[3]

The relationship of FCL-1 to the other major proteins secreted by FCL or FBS fibroblasts was also examined. FCL-1 was not precipitated by antibodies against either type I procollagen or 43K protein. Immunoblotting under denaturing conditions with antibodies to type I collagen and type III procollagen also revealed no reactivity toward FCL-1 (data not shown). Since antibodies to type I collagen and type I procollagen reacted on the blot with all the CNBr peptides of type I collagen (except those which could not be resolved on a 10% SDS–polyacrylamide gel), it is unlikely that FCL-1 represented a fragment of type I collagen in these biosynthetic experiments.

Structure of FCL-1. (*a*) *Molecular Weight under Nondenaturing Conditions.* An earlier study reported a chain molecular weight of approximately 13 000 for FCL-1 on the basis of the mobilities of type I collagen CNBr-derived peptides on SDS–PAGE (Sage et al., 1983a). When globular protein standards were used, FCL-1 migrated on SDS–PAGE as a broad band near carbonic anhydrase (M_r 30 000). Since the hydroxyproline to proline ratio in this collagen is approximately 0.4 to 1 (Sage et al., 1983a), the correct molecular weight is probably in the range of 20 000. Attempts to obtain a molecular weight for the native FCL-1 molecule by PAGE (acid–urea gels or Laemmli gels without SDS and urea) were unsuccessful, since conditions under which the type I collagen α1 and α2 chains migrated into the gel but did not separate could not be achieved. We therefore used Sephadex G-100 in Tris–saline since lathyritic rat skin type I collagen could be eluted in the void volume as triple-helical molecules that were resistant to pepsin (not shown). FCL-1 eluted in a position midway between the standard peptide markers α1–CB8 and α1–CB3. When the column buffer was changed to 0.1 N acetic acid, these relative positions were maintained (not shown). These data indicate that FCL-1 did not behave as a higher molecular weight aggregate or polymer under these chromatographic conditions.

(*b*) *Glycoprotein or Proteoglycan Nature of FCL-1.* To examine whether mannose or glucosamine was covalently associated with FCL-1, FBS cells were incubated with these radiolabeled compounds, and the culture medium proteins were fractionated as described above. Several proteins including

[3] H. Sage and R. Mecham, unpublished results.

the 43K protein incorporated both mannose and glucosamine. However, even after prolonged exposure of the gel, there were no radioactive bands comigrating with the FCL-1 standard (data not presented). As a further test for the presence of carbohydrate, purified [^{3}H]proline-labeled FCL-1 was incubated with chondroitinase ABC and with neuraminidase. If a minimum of 10–20% of the molecular weight of FCL-1 were contributed by these compounds, a slight shift in the molecular weight of FCL-1 could be observed on SDS–PAGE after enzymatic cleavage. In fact, Vaughan et al. (1985) have shown a decrease of 50 000 in the molecular weight of PG-Lt, a proteoglycan recently identified as type IX collagen, after the release of 4-sulfated and 6-sulfated disaccharides by chondroitinase ABC or AC. Neither enzyme caused a detectable decrease in the apparent molecular weight of FCL-1 on SDS–PAGE. It therefore appeared that FCL-1 was predominantly neither glycoprotein nor proteoglycan in character.

(*c*) *Proteolysis with Trypsin, Collagenase, and Pepsin.* FCL-1 had previously been described as an unusually protease-resistant protein (to trypsin, SV8 protease, mast cell protease, and proteinase K) but was readily cleaved by bacterial collagenase (Sage et al., 1983a). To confirm and extend these studies, FCL-1 purified from several different cell strains, in some cases by molecular sieve (Sephadex G-100) chromatography, was incubated with trypsin and collagenase as shown in Figure 4 (left panel). At an approximate enzyme to substrate concentration of 1:10 (lane T) or 1:1 (lane Tx), FCL-1 was not cleaved by trypsin under conditions that produced nearly complete cleavage of the 43K protein (43K, lane T). In contrast, incubation with bacterial collagenase (lane C) produced a fragment [indicated by arrow on the right side of Figure 4 (left panel)] of approximately M_r 8000 (by collagen standards) or 17 000 (by globular standards). The 43K protein was completely refractory to this enzyme (43K, lane C).

Although the collagenase cleavage product of FCL-1 (M_r 8000) was stable over extended periods of time, the lack of complete cleavage as shown in the left panel of Figure 4 dictated changes in reaction conditions for this protein. Extension of the incubation time or increasing the enzyme concentration were both effective in producing complete cleavage of this preparation of FCL-1 (Figure 4, right panel, lanes 1–3). As the relative distribution of proline in FCL-1 is not known, we cannot state with certainty whether (a) the collagenase-resistant peptide(s) of M_r 8000 represented an end product of the reaction (but contained fewer [^{3}H]proline residues than the collagenase-sensitive portion of M_r 13 000) or (b) the enzyme eventually would degrade all the FCL-1 into peptides that are too small to be resolved on the SDS gel. In the latter case, the M_r 8000 fragment could represent a domain of FCL-1 that is more resistant to collagenase than the rest of the polypeptide chain.

Preliminary studies had also suggested that FCL-1 exhibited a limited susceptibility to pepsin (Sage et al., 1983a). This property is atypical of collagens with uninterrupted triple helical sequences such as the interstitial types I–III and type V but is more reminiscent of types IV and VIII, which contain pepsin-sensitive sites (Sage et al., 1983b). FCL-1 was cleaved by pepsin at an approximate enzyme to substrate ratio of 1:2, with a concomitant decrease in molecular weight of 1000–2000 (Figure 4, right panel, compare pepsin © lane with lane 4). With a 10-fold increase in enzyme concentration, FCL-1 was recovered in lesser amounts, although peptides smaller than the M_r 11 000–12 000 fragment were not seen (Figure 4, right panel, lane 5). Under these conditions, type I procollagen, which was present in the FCL-1 preparation, was cleaved to

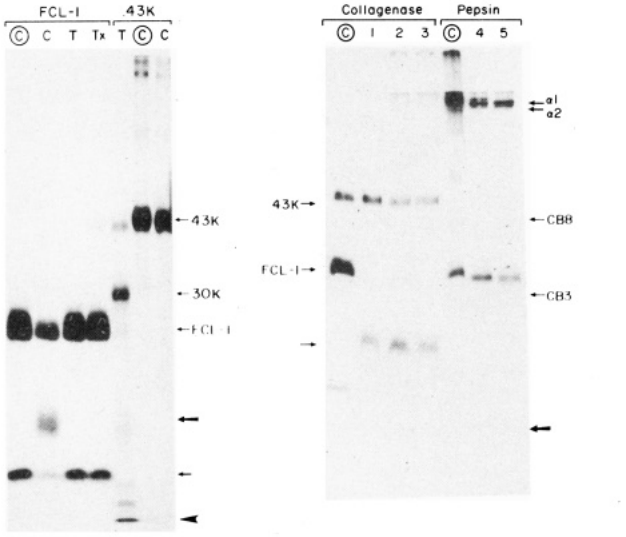

FIGURE 4: Structural analysis of FCL-1 by limited proteolysis: trypsin, collagenase, and pepsin. (Left panel) FCL-1 and a noncollagen control, 43K protein, were dissolved in Tris–saline and incubated at 37 °C with the proteases as described. Reactions were terminated by the addition of an equal volume of Laemmli sample buffer containing 100 mM DTT. The reaction products were resolved by SDS–PAGE on a 6%/12% composite gel followed by fluorescence autoradiography. ©, control digest containing no enzyme; C, bacterial collagenase (0.5 μg, 30 min); T, trypsin (0.2 μg, 20 min); Tx, trypsin (2 μg, 20 min). The 43K protein and FCL-1 have been identified. Trypsin cleavage of 43K protein produced a stable fragment of M_r 30 000, as shown (30K). Collagenase cleavage of FCL-1 (M_r 30 000, calculated from globular standards) produced a fragment(s) of M_r ~17 000 (according to globular standards), as indicated by the large unlabeled arrow. Small arrow indicates a collagenous fragment of M_r 4000. Bottom of gel is shown by arrowhead. (Right panel) For bacterial collagenase digestion, a fraction from DEAE-cellulose chromatography containing FCL-1 and 43K protein was dissolved in Tris–saline, and equal aliquots of 35 500 cpm each were incubated at 37 °C as described. Reactions were terminated by the addition of an equal volume of Laemmli sample buffer containing 100 mM DTT. For pepsin digestion, a sample containing both FCL-1 and type I procollagen was dissolved in 0.1 N acetic acid at 0 °C, and equal aliquots of 24 050 cpm each were incubated at 0 °C as described. Reactions were terminated by the addition of 1 or 10 μg of pepstatin followed by immediate transfer to dry ice and lyophilization. Reaction products were analyzed by SDS–PAGE on a 7.5/15% composite gel under reducing conditions followed by fluorescence autoradiography. ©, control incubation without enzyme. Collagenase: 1, 0.25 μg for 2 h; 2, 0.5 μg for 30 min; 3, 0.5 μg for 1 h. Pepsin: 4, 1 μg for 2 h; 5, 10 μg for 1 h. The 43K protein, FCL-1, and the α1 and α2 chains of type I collagen have been identified. The positions of elution of α1(I)–CB8 (M_r 25 000) and α1(I)–CB3 (M_r 12 000) are shown. Unlabeled arrow denotes a stable collagenase digestion product of approximate M_r 22 000. Heavy arrow indicates bottom of gel.

molecules composed of α-chains (lanes 4 and 5, α1 and α2).

Since we were interested both in the extent of triple-helical sequence and in the degree of native conformation present in these preparations of FCL-1, additional studies were performed to examine the sensitivity of both FCL-1 and type I collagen CNBr-derived peptides to pepsin. These results are summarized in Figure 5. At an approximate enzyme to substrate ratio of 1:5, FCL-1 was progressively degraded over a period of 1–6 h, under conditions in which type I collagen remained as stable, triple-helical molecules. Coomassie blue staining of the radioactive gel shown in Figure 5 showed that pepsin caused extensive degradation of the BSA present in the FCL-1 sample but that the CNBr peptides of type I collagen, especially α1–CB8, α1–CB7, and α1–CB6, were stable to the enzyme (data not presented). The larger CB peptides were more labile (e.g., α2–CB3–5) as well as those of M_r <13 000 (e.g., α1–CB3) (not shown). The data suggest that there is a range of critical length in certain triple-helical sequences

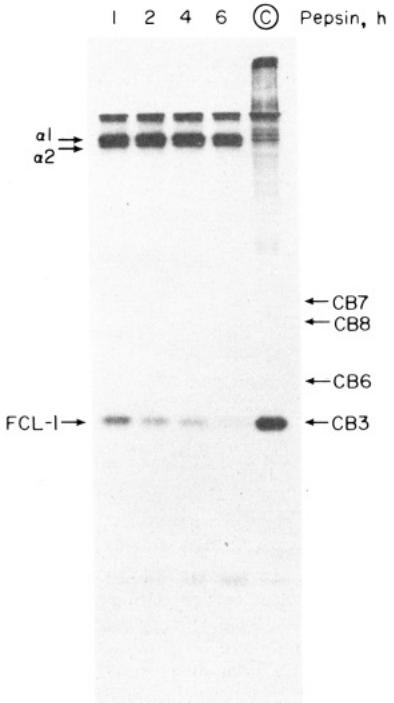

FIGURE 5: Susceptibility of FCL-1 to pepsin. FCL-1 (as shown in Figure 3B, pepsin ©) was dissolved in 0.5 N acetic acid at 0 °C. To equal aliquots of this sample (23 000 cpm) was added 10 μg of pepsin, and the reactions proceeded at 0 °C for the times indicated on the figure. Digestions were terminated by the addition of 10 μg of pepstatin followed immediately by freezing on dry ice and lyophilization. The entire reaction mixture was analyzed by SDS–PAGE on a 5%/12.5% composite gel in the absence of DTT. Fluorescence autoradiography of FCL-1 digests: ©, control incubation at 0 °C with no enzyme. FCL-1 and the α1 and α2 chains of type I collagen have been identified. The positions of migration of several CNBr (CB) peptides are shown.

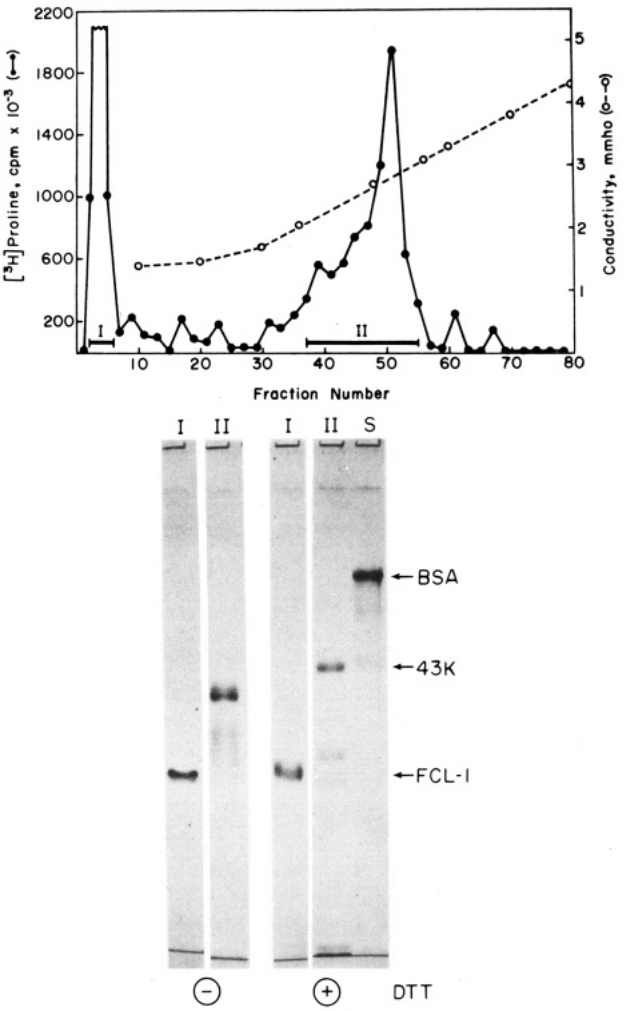

FIGURE 6: Purification of FCL-1 by CM-cellulose chromatography under denaturing conditions. A fraction from DEAE-cellulose containing FCL-1 (e.g., Figures 1 and 2, fraction IV) was denatured for 10–15 min at 40 °C and chromatographed on CM-cellulose in 6 M urea at 42 °C. (Top panel) Elution profile; Roman numerals indicate pooled fractions (peak I eluted before inception of the gradient). (Bottom panel) Fluorescence autoradiograph of column fractions analyzed by SDS–PAGE on a composite 6%/12% gel under both reducing and nonreducing conditions. Roman numerals correspond to pooled fractions shown in the top panel. S, protein eluted from the column with 0.5 M NaCl, corresponding in molecular weight to BSA.

over which collagenous sequences are refractory to pepsin cleavage. We are unable to predict the extent to which the CNBr-derived peptides renatured under our experimental conditions; however, the initial resistance of FCL-1 to a large excess of pepsin, followed by a gradual degradation without the production of discrete fragments, was similar to the behavior of α1–CB3 under similar conditions. Due to the decreased length of the FCL-1 molecule, compared to the other pepsin-resistant collagens, direct physical measurements will be necessary to determine the extent of triple-helical conformation in FCL-1.

Purification of FCL-1 by CM-cellulose Chromatography. Since the preparations of FCL-1 contained variable amounts of type I procollagen, 43K protein, and BSA [see Figure 2 (lane IV-C)], additional purification was attempted on a CM-cellulose column under denaturing conditions (42 °C, in 6 M urea). As shown in Figure 6 (top panel), the elution profile consisted of an unbound (peak I) and a bound fraction (peak II). Analysis of these fractions by SDS–PAGE is shown in the bottom panel of Figure 6. FCL-1 was not retained on the CM-cellulose column and was selectively eluted in peak I. Peak II contained the 43K protein (Figure 6, bottom panel, lanes II ± DDT), and BSA was eluted from the column with 0.5 M NaCl (lane S). The chromatographic behavior of FCL-1 on CM-cellulose under denaturing conditions was different from that of the other described collagen types (Sage & Bornstein, 1982) and provided a means by which FCL-1 could be purified for further analytical studies.

Pulse–Chase Studies of FCL-1 Synthesis. After a 1-h pulse, the [^{3}H]proline-labeled proteins secreted into the culture medium by FCL 85 cells were precipitated by ammonium sulfate (0–20% and 20–50% concentration ranges, respectively) and were subsequently incubated with bacterial collagenase. In Figure 7A are shown the SDS–PAGE analyses of the 0–20% and 20–50% ammonium sulfate fractions, prior to collagenase treatment. With increasing chase time, the proα1 and proα2 chains of type I procollagen were processed to α1 and α2 chains, respectively (compare 2 h with 15 h in the 0–20% fraction). This sort of extracellular processing, with subsequent diminution of radiolabel, could also be seen in the residual type I procollagen in the 20–50% fraction. The 43K protein (20–50% fraction) disappeared rather abruptly from the culture medium between 4 and 8 h (different secretion kinetics for this protein, however, were observed in bovine aortic endothelial cells).[4] In contrast to these two secreted proteins, FCL-1 both remained at very nearly the same intensity (assessed by incorporation of [2,3,4,5-^{3}H]proline from 2 through at least 8 h) and did not appear to undergo any

[4] H. Sage, J. Tupper, and R. Bramson, unpublished results.

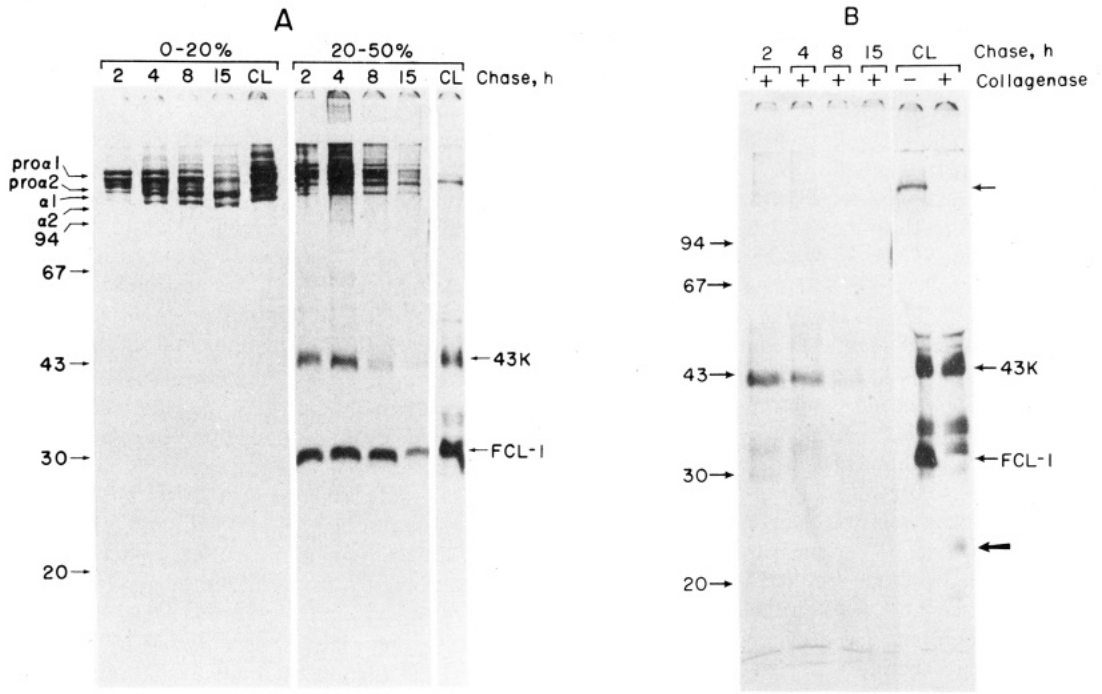

FIGURE 7: Pulse–chase analysis of the secretion of FCL-1 by FCL 85 fibroblasts in vitro. Dishes of 5–150 mm of nearly confluent FCL 85 cells (passage 4) were pulsed for 1 h with 100 μCi of L-[2,3,4,5-^{3}H]Pro/mL in serum-free DMEM containing ascorbate and β-APN. One dish retained the radiolabeling medium for an additional 15 h (continuous label, CL). The remaining four dishes were incubated in fresh medium without radiolabel supplemented with 20 mM proline. At the times indicated in the figure, the culture media were removed and the proteins fractionated in the presence of protease inhibitors by precipitation in the ranges 0–20% and 20–50% ammonium sulfate, respectively. (A) SDS–PAGE analysis of 0–20% and 20–50% ammonium sulfate precipitates of culture medium proteins. The distribution of radiolabeled proteins secreted into the medium at the chase times indicated in the figure [2–15 h, CL (continuous label)] is shown in the autoradiograph of a 5%/10% composite gel under reducing conditions. For the 0–20% fraction, 1/5 of the total recovered protein cpm from each chase time point was applied to the gel; the CL lane represents $^1/_{100}$ of the total cpm. For the 20–50% fraction equal aliquots from each chase time point, representing $^1/_2$ of the total recovered protein cpm, was applied to the gel: 2 h = 26 000 cpm, 4 h = 38 750 cpm, 8 h = 24 500 cpm, and 15 h = 18 250 cpm. The CL lane represents $^1/_8$ (156 900 cpm) of the total recovered cpm. FCL-1 and 43K protein have been identified, as well as the proα and α chains of type I collagen. The mobilities of globular protein molecular weight standards are indicated on the far left of the figure. (B) Collagenase digests of pulse–chase samples containing FCL-1. Equal aliquots of the 20–50% ammonium sulfate precipitates for each chase time point, equivalent to those shown in (A), were dissolved in Tris–saline and incubated with 0.5 μg of bacterial collagenase for 3 h at 37 °C. The CL lanes [(+) and (−) collagenase] each represent $^1/_4$ of the total recovered cpm. Reactions were terminated by the addition of an equal volume of Laemmli sample buffer containing 50 mM DTT, and the products were resolved by SDS–PAGE on a 5%/10% composite gel followed by fluorescence autoradiography. Chase times are indicated; CL, continuous label. FCL-1 and 43K protein have been identified. The unlabeled arrow denotes the α2(VI) collagen chain, and the heavy arrow shows the collagenase-resistant peptide of FCL-1. The mobilities of globular protein molecular weight standards are shown on the far left of the figure.

extracellular processing [Figure 7A, 20–50% fraction, compare 2–15 h with continuous label (CL)]. Since additional studies have shown that very small amounts of FCL-1 were first detected in the culture medium 60 min following a 1-h pulse (not shown), a synthesis and secretion time for FCL-1 was estimated to be 60 min. Shorter pulse times are necessary for a more precise determination of this secretion parameter for FCL-1; however, this collagen is synthesized in such small amounts, relative to type I, by the FCL cells that we have found the 1-h pulses necessary for the recovery of sufficient cpm for subsequent analyses.

FCL-1 (or an apparent precursor) was not observed in the 0–20% ammonium sulfate precipitate from the culture medium (Figure 7A, 0–20% fraction). Comparison of FCL-1 at 2 h with FCL-1 secreted during a continuous 15-h label (CL) showed no significant difference in molecular weight (Figure 7A, 20–50% fraction) or in disulfide bonding (data not shown). To confirm the collagenous nature of the FCL-1 band in these experiments, collagenase digestions were performed. The results, shown in Figure 7B, confirm that the radioactive bands identified in Figure 7A as type I procollagen or collagen chains and as FCL-1 (see protein standard of M_r 30 000) were sensitive to this enzyme; the 43K protein was not cleaved and served as an internal control for the digestion. With respect to the proteins secreted during the continuous label, both type VI collagen (unlabeled arrow) and FCL-1 were sensitive to bacterial collagenase while the 43K protein remained uncleaved (compare CL lanes ± collagenase).

SDS–PAGE analysis of the cell layer fractions produced the following results: (a) FCL-1 was not seen in the NaCl-insoluble fraction and (b) a collagenase-sensitive band of M_r 30 000 was not found in the NaCl-soluble extract of the cell layer. It is possible that a 10-min pulse with [^{3}H]proline was not adequate for the production of FCL-1 with a specific activity high enough to allow detection by our techniques. If FCL-1 were synthesized as a higher molecular weight precursor, it would, in addition, be difficult to distinguish between it and the major excess of type I procollagen chains which are present in the cells. A technique for enrichment for FCL-1, as has been utilized with the culture medium, is necessary to follow the biosynthetic processing of FCL-1 in cellulo. The production of antisera against this protein will facilitate these studies.

Discussion

Biosynthetic Modulation of FCL-1 Synthesis. Fetal calf ligament fibroblasts modulate the synthesis of several of their extracellular matrix proteins as a function of gestational age. Mecham et al. (1981) first described a gradient of elastin production in these cells; synthesis was significantly higher in

those fibroblasts cultured from tissue corresponding to the third trimester of development (180–270 days), with the maximum levels achieved shortly prior to birth. These investigators also demonstrated a reduction in collagen synthesis between days 110 and 270. Transcriptional regulation of the synthesis of both of these matrix proteins was indicated by the concomitant increase (in the case of elastin) or decrease (in the case of type I procollagen) in mRNA levels in the developing sheep nuchal ligament (Davidson et al., 1982). It was therefore of interest that a novel and unusual collagen, FCL-1, was also synthesized in different amounts by FCL cells from the first through the third trimester of fetal development. From several sets of experiments, the levels of FCL-1 varied from 22% in first trimester cells and 16% in second trimester cells to 7.7% in third trimester cells (Table I). This percentage is based on that fraction of culture medium proteins that precipitated between a 20 and 50% concentration of ammonium sulfate and has been used to assess relative changes in FCL-1 production by these cell strains. When measured as percent of total [^{3}H]proline-labeled protein secreted into the culture medium, FCL-1 comprised 4.4% of 95-day cellular output and 2.4% of 250-day cellular output. The declining levels of FCL-1 with increased fetal age therefore appear to parallel the changes in the synthesis of type I procollagen as described by other investigators (Davidson et al., 1982; Mecham et al., 1981).

In a preliminary report, it was suggested that FCL-1 was developmentally regulated and preferentially synthesized by cells from fetuses of later gestational age (Sage et al., 1983a). The apparent discrepancy between these earlier data and the experimental results presented here is due primarily to two factors: in the previous study, (a) FCL 150 and 270 cells were at different passages, and (b) only second and third trimester cells were analyzed. It was not until cultures of first trimester cells became available that the significant increase in the production of FCL-1 by 85- and 95-day fibroblasts, as compared to mid-trimester and late trimester cells, became apparent. Estimates of FCL-1, as percent of total protein, might also be expected to vary as a function of the isotope used to label the cells. In this regard, both [^{3}H]Pro and [^{3}H]Gly were utilized in the initial study, compared to [^{3}H]Pro in the present one.

The level of FCL-1 synthesis was also examined as a function of cellular passage in vitro and of the substratum upon which the cells were grown. There was a decrease both in total protein synthesis (as measured by incorporation of [^{3}H]Pro into secreted protein) and in FCL-1 with successive subcultivation (Table I). A similar decrease in elastin synthesis with increased cellular passage and age in culture was described by Mecham et al. (1981), in which differentiated cells at passage 6 produced 50–60% less elastin than primary cultures. Successive trypsinization also diminished the cellular biosynthetic response to dexamethasone (Mecham et al., 1984b) and to an otherwise inductive nuchal ligament substratum (Mecham et al., 1984a). Collectively, these data suggest that FCL fibroblasts gradually lose their differentiated phenotype as a function of cellular age (passage) in vitro, as assessed by the synthesis of both elastin, preferentially in late trimester cells, and FCL-1, in cells representing primarily earlier gestational ages. The presence of ligamentum nuchae ECM, whether as intact tissue minces or as isolated substrata upon which FCL cells were cultivated, stabilized the differentiated cellular phenotype with respect to elastin synthesis (Mecham et al., 1981). Induction of both elastogenic differentiation and FCL-1 synthesis has recently been demonstrated by plating cells of early gestational age on various ligament matrices (Mecham et al., 1984a).[3] A marked increase in the synthesis of FCL-1 was observed when FCL 85 cells were allowed to accumulate a substantial biosynthetic ECM. As shown in Table I, FCL-1 comprised 32% of the [^{3}H]Pro-labeled culture medium protein (20–50% ammonium sulfate precipitate) and 12.9% of the total protein. Since the distribution of FCL-1 between the culture medium and the cell layer is likely to be different for cells grown on an ECM as compared to a plastic substratum (Mecham et al., 1984a), it is possible that even higher levels of this collagen were initially synthesized and subsequently deposited on the ligamentous matrix.

Structure of FCL-1 and Comparison to Other Collagens. Several unusual features of the structure of FCL-1 are (a) a polypeptide chain of M_r 13 000, (b) resistance to human skin collagenase, mast cell protease, trypsin, staphylococcal V8 protease, and proteinase K at 37 °C, but limited susceptibility to trypsin after heating at 80 °C, and (c) comparatively high solubility in ammonium sulfate and enhanced binding to cation-exchange resins (Sage et al., 1983a). In addition, FCL-1 had a Hyp to Pro ratio of 0.43 and was cleaved partially by bacterial collagenase and by pepsin (Sage et al., 1983a). In the present study we have extended these observations and identified an M_r 8000 collagenase-resistant peptide (or, M_r 17 000 on the basis of globular protein standards, with a corresponding value of approximately 30 000 for the intact FCL-1 chain) (Figure 4). In contrast, incubation with pepsin, under conditions that produce α chain containing molecules from type I procollagen, resulted in the removal of a small peptide of M_r 1000–2000 (Figure 4, right panel); higher enzyme to substrate ratios caused a more extensive general degradation of FCL-1, as discrete peptides were not recovered by SDS–PAGE (Figure 5). The presence of a relatively short, non-triple-helical sequence at either one or both ends of a pepsin-resistant, possibly triple-helical molecule is reminiscent of type X collagen, which is cleaved by pepsin to M_r 45 000 fragments from the non-disulfide-bonded M_r 59 000 parent α chains (Gibson et al., 1981; Schmid & Conrad, 1982). Both the M_r 59 000 and M_r 45 000 forms of type X collagen were found to be unusually stable, with a denaturation temperature of approximately 47 °C (Schmid & Linsenmayer, 1985). FCL-1 was also highly resistant to degradation by trypsin (Figure 4, left panel; Sage et al., 1983a), an enzyme that acts rapidly on misaligned or nontriple-helical type I and type III procollagens but cleaves the propeptide regions rather slowly (Bruckner & Prockop, 1981). Although neither trypsin nor pepsin resistance provides an adequate estimate for the extent of triple-helical sequence in the shorter chain collagens, it is likely that FCL-1 contains a large proportion of this type of conformation.

The initial overall resistance to pepsin degradation suggested that the collagenous sequences of FCL-1 were not interrupted by noncollagenous domains, in contrast to type IV and possibly type VIII collagens which contain discrete insertions of globular regions within the triple helix (Bornstein & Sage, 1980; Sage et al., 1983b). In this regard, FCL-1 is also clearly different from type VI collagen, a newly described, highly unusual type with a constituent chain length of $M_r \sim 140\,000$ that is only 30% collagenous (von der Mark et al., 1984, and references therein). Yet another molecular arrangement of Gly-X-Y and noncollagenous domains has been described for cartilage type IX collagen, which lacks the N- and C-terminal propeptides that are characteristic of the fibrillar collagen types I–III but contains noncollagenous regions within a long triple helix (Lozano et al., 1985; van der Rest et al., 1985).

Other characteristics also indicated that FCL-1 was likely to be a structurally unique collagen. (a) FCL fibroblasts incorporated neither [^{3}H]mannose nor [^{3}H]glucosamine into FCL-1, and the protein was unaffected by neuraminidase and chondroitinase ABC. Many of the collagen types contain complex carbohydrate, and one of the chains of type IX collagen is covalently linked to glycosaminoglycan (Vaughan et al., 1985). (b) Immunoblotting and/or ELISA with polyclonal antibodies to type I and type III procollagen, type I collagen, and type IX collagen (H. Sage and G. Balian, unpublished experiments) did not reveal any cross-reactivity toward FCL-1. (c) FCL-1, as recovered from either skin or ligament fibroblast culture media, contained intrachain but not interchain disulfide bonds (Figure 2). (d) Molecular sieve chromatography under native conditions revealed a molecular weight for FCL-1 that was only slightly higher than that observed by SDS–PAGE [and similar to that of α1(I)–CB3], an indication that FCL-1 did not form aggregates or polymers under these conditions.

Biosynthetic (pulse–chase) studies have shown that FCL-1 was secreted within 60 min after a 1-h pulse with [^{3}H]Pro and did not exhibit extracellular processing within 15 h (Figure 7). This synthesis and secretion time represents a maximum estimate, as shorter labeling times from which adequate amounts of FCL-1 could be recovered were not experimentally feasible. We also found that the incorporation of [^{35}S]Met into FCL-1 was quite low, relative to other proteins. The fibrillar collagens (types I and III) produced by FCL cells exhibited a more rapid secretion and a stepwise extracellular processing [Figure 7A; see Bornstein & Sage (1980) for a review]. In contrast, at least three collagen types (IV, VI, and VIII) exhibited a more delayed secretion with limited, if any, conversion ex cellulo (Sage et al., 1983b; von der Mark et al., 1984, and references therein). Since the kinetics of secretion of extracellular molecules in vitro are partially a function of primary structure and conformation, analysis of the genes encoding these collagens will be necessary before the biosynthetic regulation and secretion can be properly understood.

Relationship to Microfibrillar Protein and Development. Most vertebrate elastic tissues contain 11–14-nm microfibrils that are associated primarily with early stages of elastogenesis and comprise collectively a heterogeneous group of proteins [see Cleary & Gibson (1983) for a review]. Two groups have independently isolated an M_r 35000 microfibrillar component (Kawaguchi, 1982) and an M_r 34000 structural glycoprotein (Serafini-Fracassini et al., 1981) from bovine nuchal ligament which appeared similar by amino acid composition. The structural glycoprotein was sensitive to bacterial collagenase and formed 11-nm fibrils in vitro. It is unlikely, however, that either of these proteins corresponds to FCL-1 (of globular M_r 30000), as both contain sialic acid and hexose and the levels of hydroxyproline were less than 1%. FCL-1 was not recovered after sequential extraction of this tissue with 1 M NaCl–50 mM Tris-HCl, pH 7.5, buffer, 5.2 M guanidine hydrochloride, and 5.2 M guanidine hydrochloride containing 1% mercaptoethanol, followed by DEAE-cellulose chromatography, collagenase treatment, and SDS–PAGE (H. Sage, unpublished experiments). However, the function of collagen proteins as certain types of microfibrillar components should not be discounted (von der Mark et al., 1984).

The developing nuchal ligament system provides several opportunities to study the regulation of gene expression with respect to proteins that comprise the extracellular matrix. Experiments by Mecham et al. (1984a) have indicated that FCL cells of early gestational age (non-elastin producing) could be induced to synthesize elastin when grown on a ligament matrix from animals of later (elastin-producing) age. Since this homogeneous population of cells required only ECM contact to initiate elastin synthesis, the authors have concluded that the FCL fibroblasts create their own microenvironment to support such developmental changes.

Our data suggest that the low molecular weight collagen FCL-1 exhibits differential expression as a function of developmental age. At the present time, we have no information regarding the function of this novel gene product. Another low molecular weight collagen, type X, has a specific distribution in skeletal structures and was preferentially associated with subpopulations of chondrocytes in the hypertrophic zone of developing bone (Schmid & Linsenmayer, 1985). In addition, developmental regulation of two of the nematode collagen genes, *col*-1 and *col*-2, has recently been shown by Kramer et al. (1985). It is reasonable to assume that collagens of unusual structure would play critical roles in cellular recognition and association, or as inductive components in the extracellular matrix. Gross (1985) has suggested that a primordial collagen precursor might have very limited triple-helical conformation but would interact preferentially with other extracellular macromolecules. Selective pressure for the evolution of a stable triple helix capable of self-association and fibril formation may have occurred in only one class of collagen genes. Proteins similar in structure to FCL-1 are nevertheless likely to fulfill the criteria for inclusion as members of the large and multistratified collagen gene family (Ninomiya et al., 1984). In particular, FCL-1 is secreted by mesenchymal cells and therefore most probably an extracellular protein. It displays triple-helical character, contains significant amounts of hydroxyproline, and is sensitive to bacterial collagenase. The potential of aggregate formation with other components of the extracellular milieu is a topic of current investigation.

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Inhibition of DNA Replication and DNA Polymerase α Activity by Monoclonal Anti-(DNA Polymerase α) Immunoglobulin G and F(ab) Fragments†

Michael R. Miller,*,‡ Charles Seighman,‡ and Roger G. Ulrich§
Department of Biochemistry, West Virginia University Medical School, Morgantown, West Virginia 26506, and Pathology and Toxicology Research, The Upjohn Company, Kalamazoo, Michigan 49001
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ABSTRACT: The effect of monoclonal anti-(DNA polymerase α) immunoglobulin G (IgG) and F(ab) fragments on DNA replication in lysolecithin-permeabilized human cells and on DNA polymerase α activity was determined. DNA polymerase α activity in vitro was inhibited equally by the same concentrations of monoclonal IgGs and F(ab) fragments. However, the IgGs and F(ab) fragments were not equally potent in inhibiting DNA replication in permeable cells. In general, the F(ab) fragments were $\simeq$10-fold more potent than IgGs in inhibiting DNA replication, suggesting the F(ab) fragments cross the nuclear membrane more readily than IgGs. Immunocytochemical studies demonstrated that at least a fraction of anti-(DNA polymerase α) IgGs entered the nucleus of permeable cells. For most antibodies tested, the IgG or F(ab) concentration needed to inhibit replication was several orders of magnitude higher than that needed to neutralize polymerase α activity extracted from the same number of cells. Anti-(DNA polymerase α) F(ab) fragments were shown to inhibit the discontinuous synthesis of Okazaki DNA, as well as the maturation of Okazaki DNA to larger DNA, thereby implicating DNA polymerase α in both of these processes.

DNA replication, DNA repair, and RNA transcription are complex processes, and a variety of different approaches have been used to study their regulation. The relative ease of obtaining and characterizing mutants in specific proteins has resulted in a beter understanding of the regulation of these processes in prokaryotes (Kornberg, 1980, 1982; Lindahl, 1982; Ogawa & Okazaki, 1980; von Hipple et al., 1984). However, due to the difficulty in obtaining specific genetic mutants in mammalian cells, other approaches are required to study the function of proteins involved in mammalian DNA repair, replication, and transcription. Inhibitors of specific mammalian enzymes have been used to study the roles of some proteins involved in these nuclear processes. For example, aphidicolin inhibits DNA polymerase α (Ohashi et al., 1978) and has been used to show involvement of this enzyme in DNA replication and DNA repair in intact cells and in subcellular systems (Ciarrochi et al., 1979; Dresler & Lieberman, 1983;

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* Correspondence should be addressed to this author.
‡ West Virginia University Medical School.
§ The Upjohn Company.

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