Collagen Synthesis by Human Skin Fibroblasts in Culture: Studies of Fibroblasts Explanted from Papillary and Reticular Dermis

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Matched human skin fibroblast cultures were established from papillary and reticular dermis. Papillary dermal fibroblasts exhibited increased plating efficiency, exponential growth, and confluent density when compared with their matched reticular dermal cultures. Collagen synthesis by these cells, however, was essentially similar regardless of their origin. Relative collagen synthesis was similar at confluent densities. No differences in type specific collagen synthesis could be detected; relative amounts of types I and III collagens in culture media and types I, III, and AB collagens in the cellular pellet were similar. Type I pC collagen was consistently elevated in culture media from reticular dermal fibroblasts when compared to papillary dermal fibroblasts. The significance of this difference in procollagen processing is unknown.

Human skin fibroblasts in culture have become an important aid in the investigation of genetic defects and in understanding the biochemical nature of cell growth, aging, and neoplastic transformation. Because these cells devote a considerable portion of their biosynthetic effort to collagen production, they are a useful system for studying connective tissue disease and regulation of collagen biosynthesis.

Recently evidence has been reported which clearly indicates that human skin fibroblasts in culture are a heterogeneous population of cells [1–4]. Indeed cells carefully explanted from papillary dermis grow faster and longer than cells from the reticular dermis [5]. Since selective factors probably affect the final population of cells in a given explant, we were curious to know if differences in collagen biosynthesis could be determined between skin fibroblasts explanted from papillary and reticular dermis.

MATERIALS AND METHODS

Normal breast skin tissue from 3 women ages 31, 58 and 63 was obtained during reductive mammoplasty. After washing with phosphate buffered saline and cutting away adipose tissue, papillary dermis with epithelium was removed at a depth of 200 μm with a Stadie-Riggs microtome (A. H. Thomas, Co.) and minced with scissors. Reticular dermis was dissected and minced by scissors from the bottom of the residual dermis. Papillary and reticular dermal minces were explanted and grown in plastic dishes (Corning) in Dulbecco's modified Eagle medium (DME) containing 20% heat inactivated (56°, 30 min) calf serum (Gibco), 100 units/ml potassium penicillin G (Squibb), 100 μg/ml streptomycin sulfate (Eli Lilly) and 25 mM HEPES (Calbiochem).

Abbreviations:
DME: Dulbecco's modified
DDT: dithiothreitol

Cells were incubated in humidified 5% CO_2-95% air at 37°C. Media was changed twice weekly. Cells were harvested in 0.05% trypsin—0.02% EDTA and counted in a Coulter counter.

Cell Growth and Labeling Method

Fibroblasts were plated in 60-mm plastic dishes at a density of 1.0 x 10^5 cells/plate and grown for 1–8 days. Plating efficiency was determined at day 1. Mean population doubling time was calculated from the slope of the line obtained during exponential growth (days 2–5). Final density at confluence was arbitrarily measured at day 8. Cells were compared at the same passage level.

Labeling conditions were patterned after those previously shown to yield optimal collagen synthesis [6]. Confluent fibroblasts were preincubated for 4 hr and during labeling with DME as above except serum was 10% dialyzed calf serum and additions included sodium ascorbate (30 μg/ml), glucose (final concentration 4.5 mg/ml) and β-aminopropionitrile (25 μg/ml). Labeling was carried out in fresh medium containing 50 μCi 2,3-^{3}H-proline (New England Nuclear) for 18 hr. The following protease inhibitors were added to medium and cell wash: 1 mM N-ethylmaleimide, 1 mM phenylmethylsulfonylflouride, 1 mM EDTA.

Measurement of Relative Collagen Synthesis

Labeled media was analyzed by digestion with clostridial collagenase [6].

Pepsin Treatment of Cultured Media and Cell Pellet

Cultured media was extensively dialyzed against 0.5 M acetic acid and lyophilized. Cell pellets were extracted in 0.5 M acetic acid at 4°C for 24 hr and lyophilized. The recovery of ^{3}H-proline from cell pellets by acetic acid extraction was 73–81%. Lyophilized samples were dissolved in 0.5 M acetic acid (10 mg/ml) and digested with 100 μg/ml pepsin (Worthington) for 6 hr at 4°C. Following neutralization with NaOH, collagen was coprecipitated with carrier collagen (lathyritic rat skin collagen) by ammonium sulfate (176 mg/ml).

Ion Exchange and Molecular Sieve Chromatography

Procollagen and intermediates in procollagen conversion were isolated from media by DEAE-cellulose chromatography [6]. Types I and III collagen were separated on 6% agarose (A-5 M Bio-Rad) in 2 M guanidine hydrochloride [6] and carboxymethyl cellulose [7].

SDS-Polyacrylamide Slab Gel Electrophoresis

SDS-polyacrylamide slab gel electrophoresis and fluorography were carried out as previously described [6]. Developed gels were scanned with a Gelman ACD-5 gel scanner within the linear range.

DNA

DNA content in the cell pellet was determined by the method of Burton [8].

Intracellular Proline Pool

Cell pellets were washed 3 times with phosphate buffered saline. Cells were disrupted in 0.5 ml distilled water by freezing and thawing 3 times. After precipitating the protein with an equal volume of trichloroacetic acid, the supernatant was desalted on Dowex AG-50 x 8 (100–200 mesh), eluted with 2 M NH_4OH and evaporated [9]. Proline was separated on a Beckman Model 119 amino acid analyzer. An aliquot was counted in Aquasol-2 (New England Nuclear) in a Beckman LS-9000 liquid scintillation counter. The amount of free proline was determined by reaction with ninhydrin [10].

RESULTS AND DISCUSSION

Cell Growth and Relative Collagen Synthesis

Papillary dermal fibroblasts had better plating efficiencies (1.3–1.5 times), faster doubling times (1.1–2.0 times), and more cells at confluence (1.3–3.5 times) when compared with matched reticular dermal fibroblasts, which confirms the report of Harper and Grove [5].

Labeled proline incorporation by papillary and reticular dermal fibroblasts into total protein (40,500 ± 4,600 cpm/μg DNA and 41,100 ± 5,300 cpm/μg DNA, respectively) and into collagenase sensitive protein (222,000 ± 3,800 cpm/μg DNA and 21,200 ± 900 cpm/μg DNA) was essentially similar and relative collagen synthesis (cpm of collagen/cpm of total protein) was the same (0.61 ± 0.08 and 0.54 ± 0.03). Specific activities of intracellular proline pools were similar in papillary and reticular dermal fibroblasts (29.0 ± 6.9 cpm/μg proline and 25.4 ± 6.2 cpm/μg proline, respectively).

Synthesis of Various Types of Collagen in Cultured Media and Cell Pellet

The content of type III collagen in media synthesized by papillary and reticular fibroblasts was estimated by 3 different methods (CMC and Agarose chromatography as well as SDS-slab gel electrophoresis). The recovery in the eluate from CMC and Agarose chromatography was over 80%. Densitometric scanning of type I and III collagens by SDS-slab gel fluorography was carried out within the linear range (0–3 × 10⁴ cpm/48 hr. exposure). As shown in Table I, no remarkable difference in the type III collagen content between papillary and reticular fibroblasts were found by the 3 different methods. Estimation of type III collagen content by CMC (12.5–15.7%) and Agarose (10.5–15.1%) chromatography gave higher values then that by SDS-slab gel fluorography (5.7–9.8%) as previously described elsewhere [11]; the reason for this discrepancy is not clear. Fleischmajer et al reported slightly higher relative type III collagen synthesis in reticular dermal fibroblasts when com-

TABLE I. *Relative type III collagen synthesis in pepsin treated media from papillary and reticular dermal fibroblasts*

	Cell source	CMC[a]	Agarose[a]	SDS-slab gel[a]
Case 1	Papillary	15.2	15.1	6.0
	Reticular	12.7	14.1	5.7
Case 2	Papillary	14.5	13.5 ± 0.9[b]	8.2
	Reticular	15.7	10.5 ± 0.2[b]	6.5
Case 3	Papillary	12.5	10.9 ± 0.2[b]	9.8
	Reticular	14.5	11.8 ± 1.0[b]	9.6

[a] Values represent percent of type III collagen.
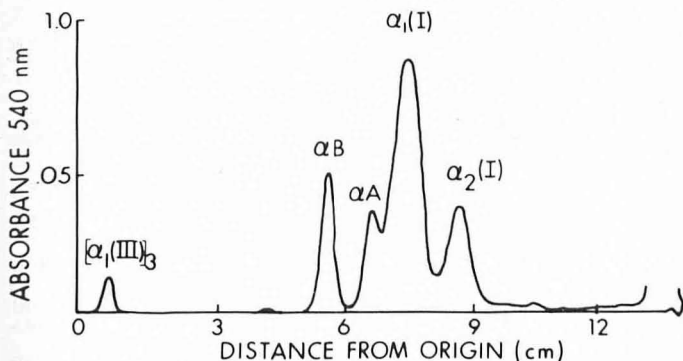[b] Values are mean values of 2 experiments.

FIG 1. Densitometric tracing of collagens isolated from cell layer of case 2 papillary fibroblasts by 0.5 M acetic acid followed by pepsin digestion as described under Materials and Methods. SDS-polyacrylamide slab gel electrophoresis was carried out without reduction.

TABLE II. *Relative amounts of types I, III and AB collagens deposited in the cell layer by papillary and reticular dermal fibroblasts[a]*

	Cell source	$[(\alpha_1(III)]_3$	αB	αA	$\alpha_1(I)$	$\alpha_2(I)$
Case 1	Papillary	4	10	4	58	24
	Reticular	2	17	15	49	18
Case 2	Papillary	3	18	11	48	20
	Reticular	2	6	10	61	21
Case 3	Papillary	4	14	12	51	21
	Reticular	4	13	11	53	20

[a] Determined by SDS-slab gel electrophoresis of pepsinized cell pellets. Values represent percent density determined from scanning of fluorograms.
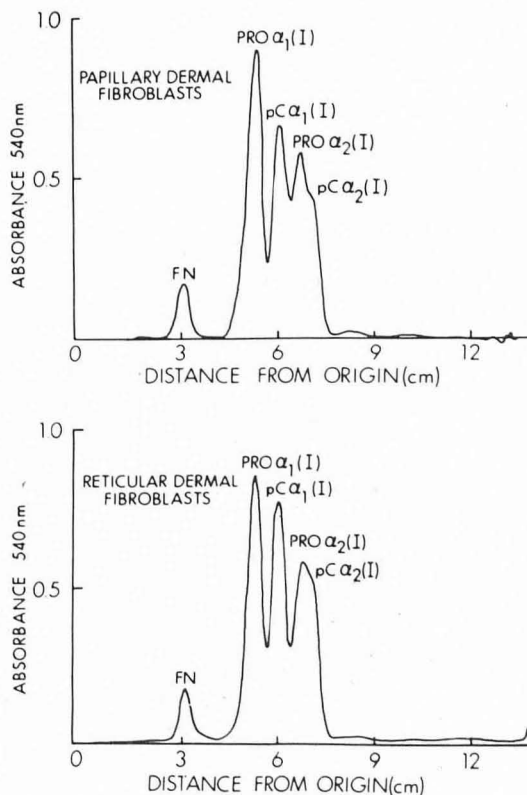

FIG 2. Densitometric tracings of radioactive culture media proteins of case 1. Culture media was dialyzed against 0.5 M acetic acid with protease inhibitors, lyophilized, and run on SDS-polyacrylamide slab gels in the presence of dithiotheitol (DTT).

pared with papillary dermal fibroblasts [12]. These results however related to fibroblasts in primary culture which may account for the difference from our results.

SDS-slab gel electrophoresis revealed that pepsinized collagens in the cell layer from papillary and reticular fibroblasts consisted of type I, III and AB collagens (Fig 1). AB collagens were identified by (a) not precipitable by 2.6 M NaCl at neutral pH, (b) not precipitable by 0.7 M NaCl at acidic pH, and (c) relatively high hydroxylysine to hydroxyproline ratio detected by amino acid analysis (data not shown). Estimation of the amounts of each chain showed no consistent differences between papillary and reticular dermal fibroblasts (Table II).

Nature of Collagens Secreted into Cultured Media

Collagenous proteins secreted into culture media from papillary and reticular dermal fibroblasts were compared by DEAE chromatography and SDS-slab gel electrophoresis. SDS-slab gel electrophoresis showed that at least 5 high molecular weight proteins were present in media (Fig 2). The proteins in bands

TABLE III. *Media proteins secreted by papillary and reticular dermal fibroblasts*[a]

	Cell Source	Fibronectin	Proα_1(I)	pCα_1(I)	Proα_2(I)	pCα_2(I)	$\dfrac{pC\alpha_1(I) + pC\alpha_2(I)}{Pro\alpha_1(I) + Pro\alpha_2(I)}$
Case 1	Papillary	4	51	18	22	6	33
	Reticular	3	46	21	21	8	43
Case 2	Papillary	4	48	24	14	9	53
	Reticular	2	44	29	14	11	69
Case 3	Papillary	5	38	26	18	12	68
	Reticular	5	34	29	17	15	86

[a] Determined by SDS-slab gel electrophoresis of media. Values represent percent determined from scanning of fluorograms.
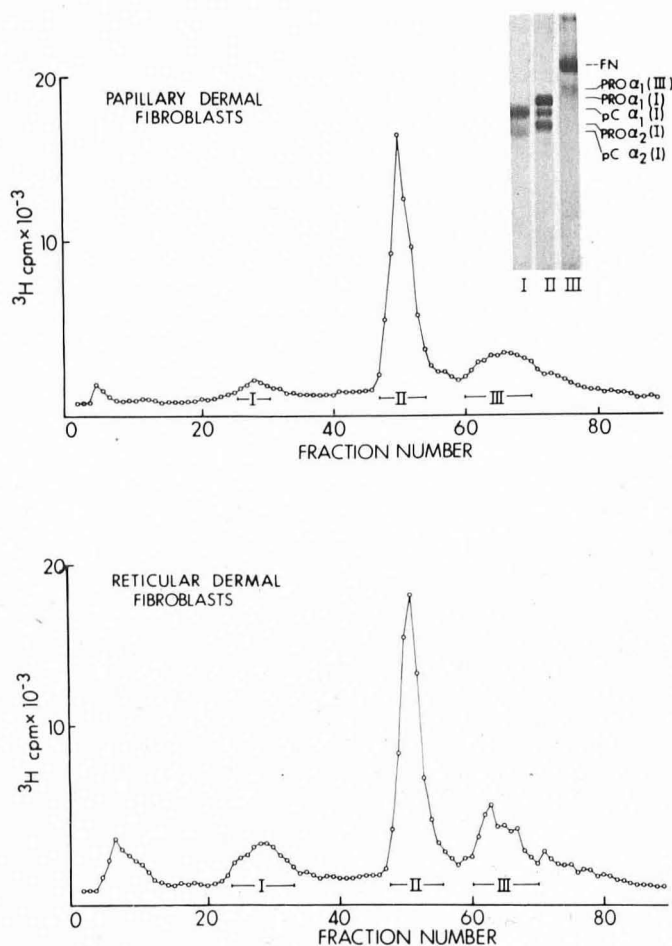

FIG 3. DEAE-cellulose chromatograms of culture media proteins from case 2. Each peak (I-III) in the gradient (tube No. 11–90) was pooled, dialyzed against 0.5 M acetic acid, lyophilized then dissolved with electrophoresis buffer. The fluorograms in insets show fraction I consists of pCα_1(I) and pCα_2(I), fraction II; Pro α_1(I), pCα_1(I), Pro α_2(I) and pCα_2(I), fraction III, fibronectin and Proα_1(III) respectively.

1–5 were identified as fibronectin, Pro α_1(I), pC α_1(I), Pro α_2(I) and pCα_2(I) respectively, by digestion with purified human skin collagenase (kindly provided by Dr. Eugene Bauer, Washington University, St. Louis, Mo.) or reduction with dithiothreitol (data not shown).

Small pNα_1(I) and pNα_2(I) bands were also detected without reduction (data not shown). The content of fibronectin was almost constant in papillary and reticular dermal fibroblast culture. The relative amount of Pc collagen however synthesized by reticular dermal fibroblasts was higher than that synthesized in papillary dermal fibroblasts (Table III). This difference was confirmed by DEAE chromatography (Fig 3). Three peaks (I-III) were obtained from both papillary and reticular fibroblasts. Peaks I-III were identified as type I pC

collagen, procollagen I + small amount of pC(I) collagen, and fibronectin + procollagen III respectively by SDS-slab gel electrophoresis (Fig 3). Peak I [pC(I) collagen] was always higher in reticular dermal fibroblasts when compared with papillary dermal fibroblasts. The difference in accumulation of pC collagen in the media in these studies appears to result in a difference in cleavage of the amino-terminal extension peptide since pN collagen chains did not accumulate in the media or cell pellet (unpublished observation). In embryonic chick tendon cells pC(I) collagen was observed to accumulate when cellular density was sparse [13]. Therefore, observed differences in confluent density between papillary and reticular dermal fibroblasts may account for the differences in pC(I) collagen accumulation detected in our study.

Although papillary and reticular dermal fibroblasts appear to be different populations of cells on the basis of cell growth characteristics, collagen synthesis in these populations of cells was similar except for pC(I) collagen accumulation. Selection could conceivably be a problem when whole skin is explanted and these studies would suggest that studies of genetic abnormalities in collagen synthesis can probably be safely undertaken in fibroblast cultures derived from whole skin, although the results are from a single site of skin and may not necessarily be extrapolated to other areas.

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