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### Research Article

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# Heterogeneity in Hormone Responses and Patterns of Collagen Synthesis in Cloned Dermal Fibroblasts

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## Abstract

Fibroblasts cultured from normal human dermis are heterogeneous with respect to growth kinetics, synthetic function, and morphologic features. There are many examples of clonal heterogeneity in apparently homogeneous connective tissue cell populations, and it has been suggested that selection of cell populations with particular phenotypic features is the basis for the development of pathologic connective tissue changes in inflammatory disorders. In these studies we report characterization of the pattern of matrix biosynthesis and responses to hormones in cells cloned from normal human dermis. The results indicate that cloned dermal fibroblasts are heterogeneous with respect to synthesis of collagens as well as their responses to prostaglandin E<sub>2</sub> and parathyroid hormone. Selective expansion of clonal populations with unique patterns of matrix synthesis and cell surface receptors could provide the basis for abnormal connective tissue remodeling in certain pathologic states. (*J. Clin. Invest.* 1990. 85:798–803.) hormone • collagen • connective tissue cell

## Introduction

Examination of the growth kinetics and morphologic features of fibroblasts cultured from neonatal human skin have demonstrated that these cells are genetically and phenotypically diverse (1–7). Examples of clonal heterogeneity in apparently homogeneous connective tissue cell populations have been reported, and it has been suggested that temporal selection of individual cell populations could be involved in cellular remodeling of connective tissues under physiologic conditions (1–9). Similar mechanisms could also be involved in pathologic states in which monoclonal or oligoclonal cell populations possessing unfavorable or inappropriate phenotypic features could be selected (10–15).

The initial goal of these studies was to determine the degree of phenotypic diversity in the pattern of matrix biosynthesis among normal skin fibroblasts. We observed significant differences in the pattern of collagen synthesis in substrains cloned from normal neonatal foreskin. These findings can be

accounted for in part, by differential regulation of collagen synthesis at the transcriptional level.

The second goal of our studies was to determine whether heterogeneity also exists in responses involving cell surface receptors for hormones known to affect connective tissue remodeling. We therefore examined the pattern and magnitude of 3'-5'-cAMP responses to PGE<sub>2</sub> and PTH. Marked heterogeneity in the cAMP responses to these hormones was also observed, although there was no correlation between the rate of collagen synthesis and the magnitude or pattern of hormone responses. Selective expansion of clonal populations *in vivo* could thus give rise to tissues with unique patterns of matrix synthesis and altered responses to hormonal regulation.

## Methods

*Processing of tissue.* Samples of neonatal foreskin obtained from routine circumcision were digested with clostridial collagenase. Substrains were isolated from primary cultures by limiting dilution cloning (1–4). Once confluent, cultures of these clones were maintained in basal Eagle's medium supplemented with FCS and carried through multiple passages after trypsinization.

*Hormone responses.* Cells were passaged into 24-well trays (Costar, Cambridge, MA) at  $\sim 5 \times 10^4$  cells/well. cAMP responses were assessed by incubating cells for 20 min at 37°C in buffer containing 1 mM 3-isobutyl-1-methylxanthine (IBMX)<sup>1</sup> (Aldrich Chemical Co., Milwaukee, WI) in the absence or presence of either PGE<sub>2</sub> or PTH. cAMP content was determined by radioimmunoassay (Becton-Dickinson & Co., Paramus, NJ) (16, 17). PGE<sub>2</sub> was a gift of the Upjohn Co., Kalamazoo, MI. Bovine PTH ( $\sim 3,000$  U/mg) extracted from TCA precipitates of bovine parathyroid glands and purified by gel filtration and ion exchange chromatography on carboxymethyl-cellulose was a gift of Dr. Henry T. Keutmann (Massachusetts General Hospital, Boston, MA).

*Labeling of medium collagens.* Cells were plated at  $2 \times 10^5$  cells/dish in 3.5-cm dishes and 2 d later incubated for 24 h with L-[5-<sup>3</sup>H]-proline (30  $\mu$ Ci/mmol) in the presence of 50  $\mu$ g/ml beta-aminopropionitrile, 50  $\mu$ g/ml ascorbic acid, and 2 mM glutamine in the absence of serum. We have previously found that under these conditions medium collagen accounts for > 95% of total collagen synthesized. Medium proteins which included procollagens and processed procollagen were analyzed by SDS-PAGE with or without reduction with 0.5%  $\beta$ -mercaptoethanol and fluorography as previously described (18). Labeled collagens were further characterized by pepsinization at 4°C followed by SDS-PAGE with or without delayed reduction to distinguish  $\alpha 1(I)$  from  $\alpha 1(III)$  collagen chains (18, 19).

*Analysis of collagen mRNAs.* Cytoplasmic RNA was extracted from cells by the method of White and Bancroft (20). After denaturation in 7.5% formaldehyde, dot hybridization was performed as described previously using cDNA probes labeled by nick translation (21, 22). The following cDNAs were used: Hf677, a 1,500-bp cDNA en-

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1. Abbreviation used in this paper: IBMX, 3-isobutyl-1-methylxanthine.

coding part of the  $\alpha 1(I)$  procollagen subunit (23), and Hf1131, a 1,500-bp cDNA encoding part of the  $\alpha 2(I)$  procollagen subunit (24) provided by Dr. F. Ramirez and Dr. D. J. Prockop. The cDNA for the  $\alpha 1(III)$  procollagen, pHCI-1, was provided by Dr. R. G. Crystal and Dr. M. Brantly, National Institutes of Health (NIH; 25). Specific activities of the labeled probes were  $\sim 2.16, 1.77,$  and  $1.45 \times 10^8$  cpm/ $\mu$ g DNA for the  $\alpha 1(I), \alpha 2(I),$  and  $\alpha 1(III)$  procollagen cDNAs, respectively.

For Northern hybridization, total RNA was extracted using the guanidine-thiocyanate procedure (21). Total RNA (5  $\mu$ g) was denatured by treatment with formaldehyde-formamide and electrophoresed on 0.8% agarose gels containing 0.22 M formaldehyde. The RNA was transferred to nitrocellulose paper and hybridized using the cDNA probes described above (21). The relative intensity of the 28S and 18S ribosomal RNA, compared with background as an index of RNA breakdown, was assessed using ethidium bromide and visualized under ultraviolet light. Nick translation, prehybridization, and hybridization with the procollagen cDNAs were performed as described (21).

## Results

We have previously shown that cells cultured from human dermis increase cAMP content in response to PTH or PGE<sub>2</sub> (26, 27). To examine the pattern of hormone-induced cAMP responses in dermal fibroblasts, 24 cell substrains cloned by limiting dilution from three neonatal foreskin samples were incubated with maximal stimulatory concentrations of PTH or PGE<sub>2</sub>. The three parent cultures of fibroblasts were responsive to both hormones. All substrains prepared from the parent cultures from two of the samples (D.W. and J.R.) increased cAMP content when tested with PTH or PGE<sub>2</sub>, although there were marked differences in the magnitude of the cAMP responses. Although cells from the third parent culture (L.T.F.) were responsive to PTH, none of the substrains increased cAMP levels in response to PTH (Table I). These substrains were, however, responsive to PGE<sub>2</sub>.

Fig. 1 shows the relationship between PTH- and PGE-induced cAMP responses in clones from sample D.W. Although there was a correlation of the PTH and PGE<sub>2</sub> responses in substrains from parent culture D.W. ( $r = 0.77$ ), there was no correlation in the responses to the two hormones in the substrains from parent culture J.R. (not shown). Patterns of responsiveness to PTH and PGE<sub>2</sub> remained stable after passage; in general, substrains exhibiting the greatest hormone-induced increases in cAMP levels continued to show enhanced sensitivity to hormonal stimulation.

Table I. Comparison of PTH-induced cAMP Response in a Parent Dermal Cell Culture and Two Representative Substrains

	cAMP		
	Parent	Substrains	
	L.T.F.	C	K
	<i>pmol/well</i>		
Buffer	1.84 $\pm$ 0.45	0.55 $\pm$ 0.13	0.45 $\pm$ 0.06
PTH	7.60 $\pm$ 1.82	0.52 $\pm$ 0.06	0.57 $\pm$ 0.04
PGE <sub>2</sub>	71.1 $\pm$ 1.84	47.7 $\pm$ 4.03	6.40 $\pm$ 0.93

Cells were incubated for 20 min at 37°C with buffer containing (1 mM) IBMX with or without PTH (10<sup>-7</sup> M) or  $\pm$ PGE<sub>2</sub> (2.5  $\times$  10<sup>-6</sup> M). Values represent mean $\pm$ SEM.

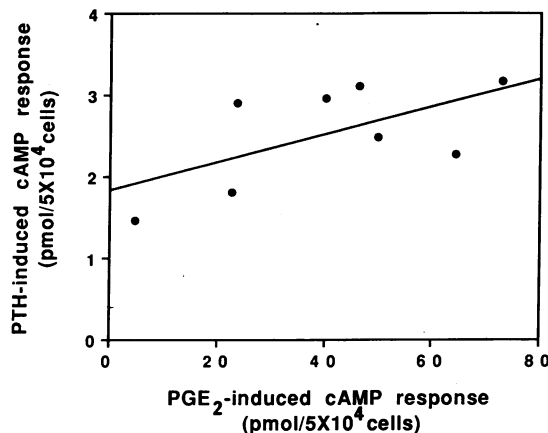


Figure 1. Comparison of the PTH- and PGE<sub>2</sub>-induced cAMP responses to maximal stimulatory concentrations of hormone in substrains from parent dermal culture D.W. Cells in 24-well trays were incubated 20 min at 37° in the presence of 1 mM IBMX $\pm$ PTH (10<sup>-7</sup> M) or PGE<sub>2</sub> (2.5  $\times$  10<sup>-6</sup> M). Points represent means of three wells for each hormone.

We have previously described an inverse relationship between endogenous PGE<sub>2</sub> production and the magnitude of the PGE<sub>2</sub>-induced cAMP response in different human connective tissue cell cultures (28–30). As shown in Fig. 2, there was no correlation between basal PGE<sub>2</sub> levels and the cAMP response to this ligand in the D.W. substrains.

To further investigate the functional heterogeneity of the dermal cells, substrains from the three separate parent cultures were examined for their capacity to synthesize collagen. All substrains produced types I and III collagen (analyzed after pepsinization and resolution of the labeled medium proteins by SDS-PAGE and fluorography), although there was variability in the amount and relative abundance of the individual collagen types. To quantitate the relative capacity of substrains to produce individual procollagens, cells were incubated with [<sup>3</sup>H]proline, and after separation of the pepsin-resistant me-

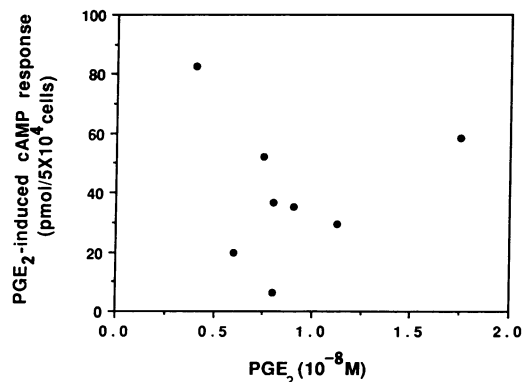
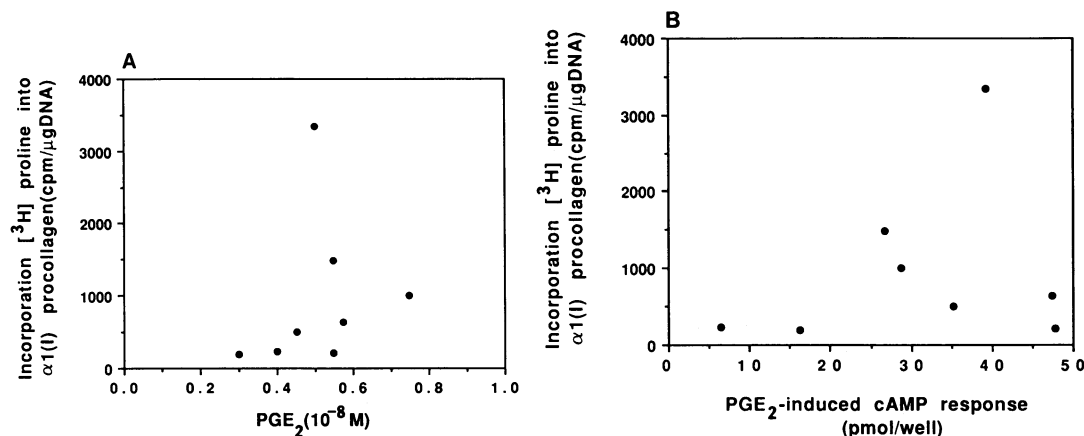


Figure 2. Comparison of the PGE<sub>2</sub>-induced cAMP response and basal PGE<sub>2</sub> production in substrains cloned from one dermal sample. Cells in 24-well trays were incubated 20 min at 37°C in the presence or absence of a maximal stimulatory concentration of PGE<sub>2</sub> (2.5  $\times$  10<sup>-6</sup> M). PGE<sub>2</sub> levels were measured after 72 h incubation of substrains (5  $\times$  10<sup>4</sup> cells) in culture medium alone. Values represent means of three wells.



**Figure 3.** Capacity of dermal substrains to incorporate [<sup>3</sup>H]proline into α1(I) procollagen; comparison with basal PGE<sub>2</sub> production and PGE<sub>2</sub>-induced cAMP response. Medium PGE<sub>2</sub> was assayed in substrains (5 × 10<sup>4</sup> cells) incubated 24 h in culture medium alone. PGE<sub>2</sub> responses were assessed by test-incubating cells (5 × 10<sup>4</sup> cells/well) for 20 min with or without PGE<sub>2</sub> (2.5 × 10<sup>-6</sup> M). *A* compares

PGE<sub>2</sub> production to α1(I) procollagen synthesis. Pepsin-resistant labeled medium proteins were separated by SDS-PAGE. The regions of the gel containing the α1(I) procollagen bands were excised and counted. Counts have been normalized to DNA content in the culture dishes at the time of labeling. PGE<sub>2</sub> production and α1(I) procollagen synthesis were negatively correlated ( $r = 0.33$ ). *B* compares α1(I) procollagen synthesis with the PGE<sub>2</sub>-induced cAMP responses, which were negatively correlated ( $r = 0.21$ ).

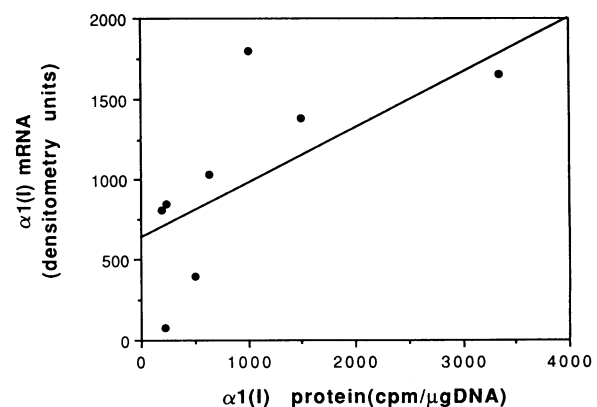
dium proteins by SDS-PAGE the labeled bands were excised and counted. Fig. 3 shows incorporation of [<sup>3</sup>H]proline into α1(I) procollagen in substrains cloned from a single sample of dermis. There was marked heterogeneity in the capacity of the substrains to synthesize α1(I) procollagen. With passage of the cells the pattern of collagen synthesis among substrains remained stable. There was no correlation between the amounts of α1(I) procollagen synthesized and the production of PGE<sub>2</sub> ( $r = 0.33$ ) (Fig. 3 *A*) or the magnitude of the PGE<sub>2</sub>-induced cAMP response ( $r = 0.21$ ) (Fig. 3 *B*).

To determine whether the heterogeneity in collagen synthesis among the substrains was correlated with steady-state levels of procollagen mRNA, cytoplasmic RNA was extracted and hybridized with labeled cDNAs for types I and III procollagen. Northern blot analysis of total RNA demonstrated that there was no cross-hybridization among the probes (data not shown). The levels of procollagen mRNA determined by dot hybridization as a function of the secreted labeled procollagen estimated by [<sup>3</sup>H]proline incorporation are shown in Fig. 4. Dot blots were quantitated by scanning with a soft laser densitometer and analyzed with an Apple IIe computer using a Zeineh Videophoresis II electrophoresis reporting program (Biomed Instruments Inc., Fullerton, CA) as previously reported (21). There was a correlation between the steady-state levels of α1(I) procollagen mRNA and incorporation of [<sup>3</sup>H]-proline into α1(I) collagen chains ( $r = 0.68$ ). No correlation was found in the pattern of collagen synthesis and hormone responsiveness.

The levels of α1(I) and α2(I) mRNAs were quantitated in substrains from one parent culture (L.T.F.) by scanning the dot blots after hybridization with cDNA probes. The ratios of the α1(I) and α2(I) procollagen mRNAs in the substrains ranged from 1.37 to 2.46. As shown in Fig. 5, the ratios of α1(I)/α2(I) mRNA were inversely correlated with the ratio of α1(III)/α1(I) plus α2(I) (type III/type I collagen). Comparison of the levels of types I and III procollagen mRNA (Fig. 6) revealed a significant positive correlation ( $r = 0.86$ ;  $P < 0.001$ ). The correlation was best between the levels of α1(III) and α2(I) procollagen ( $r = 0.89$ ;  $P < 0.001$ ) compared with that of α1(III) and α1(I) ( $r = 0.79$ ;  $P < 0.01$ ).

## Discussion

We have previously demonstrated that in response to products present in medium conditioned by PBMC, dermal fibroblast populations cultured from different individuals exhibit marked phenotypic diversity in proliferative responses and synthesis of PGE<sub>2</sub> and collagenase (1–4). This conditioned medium contains many biologically active cytokines, such as IL-1 or tumor necrosis factor, that could potentially influence the functions of connective tissue cells. We speculated that the heterogeneity in proliferative responses to these ligands could provide a mechanism for immune-mediated clonal selection at sites of inflammation by favoring overgrowth of clonal cell populations with phenotypic characteristics that promote disease expression. It has been proposed that several chronic in-



**Figure 4.** Comparison in dermal substrains of the α1(I) procollagen mRNA levels and the amount of secreted α1(I) procollagen estimated by [<sup>3</sup>H]proline incorporation. After transfer of cytoplasmic RNA to nitrocellulose and hybridization with the labeled procollagen cDNAs the autoradiographs were quantitated by densitometric scanning. Incorporation of [<sup>3</sup>H]proline into α1(I) procollagen was determined by counting excised bands from SDS-PAGE gels after separation of labeled medium proteins as described in Fig. 3. Each point represents the value for a single substrain.

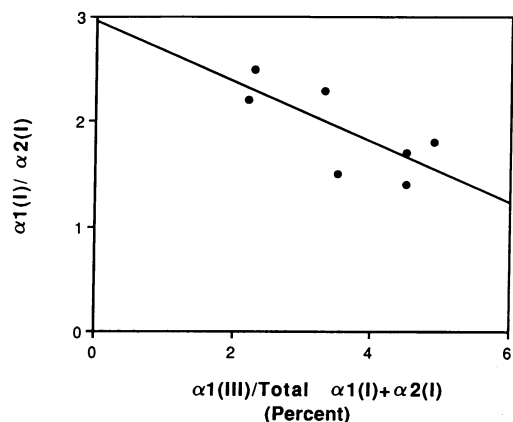


Figure 5. Comparison in dermal substrains of the ratio of  $\alpha 1(I)/\alpha 2(I)$  procollagen mRNA with the ratio of type III/type I procollagen mRNA. After extraction cytoplasmic RNA was transferred to nitrocellulose and hybridized with  $^{32}\text{P}$ -labeled  $\alpha 1(I)$ ,  $\alpha 2(I)$ , or  $\alpha 1(III)$  procollagen probes. RNA was quantitated by densitometric scanning of the autoradiographs. Each point represents the values for a single substrain.

Inflammatory conditions associated with increased connective tissue deposition such as hepatic cirrhosis, interstitial pneumonitis, nephritis, and scleroderma (7, 31–33) could be explained by selection in vivo of dominant clones of cells that proliferate and produce relatively large amounts of matrix.

In these studies we further evaluated dermal fibroblast populations by measuring the production of extracellular matrix macromolecules by substrains derived from parent cultures. Marked differences in the pattern and amounts of collagen synthesis were demonstrated. Furthermore, the differences in the amounts and types of secreted procollagens estimated by [ $^3\text{H}$ ]proline incorporation correlated with the steady-state levels of the corresponding procollagen mRNAs. Although these results are consistent with differential regulation of collagen synthesis at a transcriptional level, they could also be accounted for by differences in mRNA stability. Direct mea-

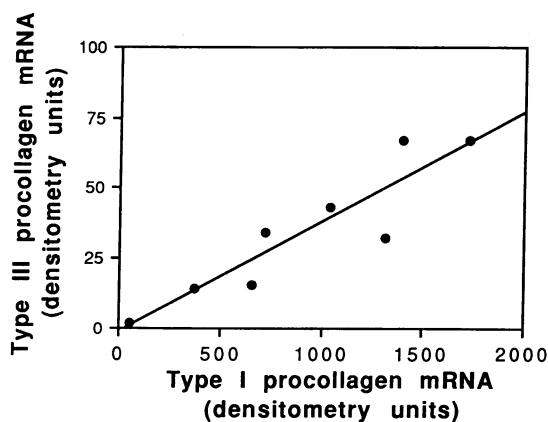


Figure 6. Comparison of the levels of type III [ $\alpha 1(III)$ ] to type I [ $\alpha 1(I) + \alpha 2(I)$ ] procollagen mRNA in substrains from parent culture L.T.F. After extraction cytoplasmic RNA was transferred to nitrocellulose and hybridized with  $^{32}\text{P}$ -labeled  $\alpha 1(I)$ ,  $\alpha 2(I)$ , or  $\alpha 1(III)$  procollagen probes. RNA was quantitated by densitometric scanning of the autoradiographs. Each point represents the values (expressed in densitometry units) for a single substrain.

surements of transcription and mRNA stability are required to establish the molecular basis for heterogeneity in matrix synthetic capacity among substrains.

We previously noted variation in the ratios of levels of  $\alpha 1(I)$  and  $\alpha 2(I)$  procollagen mRNAs in cultured human chondrocytes and synovial fibroblasts (19, 21, 22, 34). These results suggested that the expression of these procollagen genes is not coordinately regulated. We speculated that the excess  $\alpha 2(I)$  procollagen mRNA was either not translated or the translated product was not secreted by the cells. Similar variation in the ratio of the levels of the  $\alpha 1(I)$  and  $\alpha 2(I)$  procollagen mRNAs was observed among the dermal substrains in the present report, consistent with differential regulation of these two procollagen mRNAs. Furthermore, the results would indicate that the normal ratio of  $\alpha 1(I)/\alpha 2(I)$  of 2:1 in parent cultures may reflect the average of constituent populations that vary significantly from this ratio.

Miskulin et al. (25) previously reported that the expression of the type III collagen gene is modulated coordinately with that of the type I collagen genes based on comparison of procollagen mRNA levels in dividing and nondividing cells. In our studies we did not observe a correlation between collagen synthesis and cell doubling times. However, the levels of type III procollagen mRNA were strongly correlated with those of type I ( $r = 0.86$ ;  $P < 0.001$ ). This correlation was best between  $\alpha 1(III)$  and  $\alpha 2(I)$  ( $r = 0.89$ ;  $P < 0.001$ ) compared with that between  $\alpha 1(III)$  and  $\alpha 1(I)$  ( $r = 0.79$ ;  $P < 0.01$ ). Our results are consistent with coordinate regulation of types I and III collagen gene expression in these substrain populations.

Botstein et al. (7) have reported that there is marked heterogeneity in the capacity of cloned human foreskin fibroblasts to incorporate [ $^3\text{H}$ ]proline into collagens, although the specific collagen types synthesized were not characterized. More recently, Crouch et al. (35) have reported that morphologically distinct clones of a human tumor cell line exhibit heterogeneity in the production of collagen as well as fibronectin. Dermal fibroblasts cultured from patients with scleroderma produce more types I and III collagen than fibroblasts from normal subjects (36–38); this enhanced collagen synthesis is accompanied by increased steady-state levels of types I and III procollagen mRNAs. Since the scleroderma phenotype persists over many passages in vitro these observations are consistent with the selection of populations of fibroblasts in vivo which are characterized by high rates of collagen synthesis. Our findings of heterogeneity in the pattern of matrix synthesis among substrains derived from normal dermal fibroblasts provide a rationale for apparent clonal selection of fibroblasts in a disease such as scleroderma. Of interest in this regard are our earlier observations that short-term exposure of dermal cell cultures to mononuclear cell conditioned medium results in selection of cell populations with enhanced proliferative and  $\text{PGE}_2$ -synthetic responses upon reexposure to this mononuclear cell conditioned medium (1). In analogous studies, Duncan and Berman have shown that transient exposure of dermal fibroblasts to  $\gamma$ -interferon leads to a persistent decrease in collagen synthesis (39). Inflammatory cytokines may thus have long-lasting effects on the phenotype of connective tissue cells.

In this report we have also described responses of fibroblast substrains to two hormones that affect connective tissue remodeling. Since  $\text{PGE}_2$  has been shown to modulate collagen synthesis by autocrine as well as paracrine mechanisms, we were particularly interested in establishing whether or not

there was a correlation between PGE<sub>2</sub> responsiveness and the level of collagen synthesis (22, 34, 40). We had previously shown that the responsiveness of many different connective tissue cell types (including dermal-, synovial-, and bone-derived cells) to PGE<sub>2</sub> is markedly influenced by the levels of endogenous PGE<sub>2</sub> (28–30). Treatment of cultured cells with mononuclear cell products such as IL-1 $\beta$  or tumor necrosis factor- $\alpha$ , which increased endogenous PGE<sub>2</sub> synthesis, resulted in desensitization to PGE<sub>2</sub> stimulation. These results were consistent with downregulation of receptors by high ambient levels of PGE<sub>2</sub>. In the present studies we did not observe a correlation between endogenous levels of PGE<sub>2</sub> production and PGE<sub>2</sub>-induced cAMP responses. Basal unstimulated levels of PGE<sub>2</sub> were very low (1.6–7.5 ng/ml) in medium from these cells, however, and based on previous studies with skin and synovial fibroblasts (28, 29) higher levels of PGE<sub>2</sub> may be required for receptor downregulation.

We had previously reported that cells cultured from human dermis increase cAMP levels after incubation with PTH (16, 26, 27). We demonstrated by photoaffinity labeling that the PTH receptor in these cells was similar to that in other PTH target tissues (bone and kidney) (41). Others have confirmed our observations concerning PTH responsiveness in dermal cultures, although the kinetics of PTH binding may differ in skin- and bone-derived cells (42–44). We were interested in examining the cells for responses to PTH, since this hormone may also modulate collagen synthesis (possibly by inducing increases in intracellular cAMP levels) (45, 46). Our present results indicate that, similar to PGE<sub>2</sub>, the substrains exhibit marked heterogeneity in their responsiveness to PTH. In fibroblast substrains obtained from one sample, PTH and PGE<sub>2</sub> were positively correlated, but this was not observed in substrains from another sample. These results favor heterogeneity in cell surface receptor function rather than a generalized up- or downregulation of adenylate cyclase activity. We have not examined the substrains for the capacity to produce the recently described PTH-related peptide (47–51). This ligand does increase cAMP levels in PTH-responsive tissues including dermis, and could potentially downregulate PTH receptors in a manner analogous to endogenous PGE<sub>2</sub>.

One factor potentially contributing to the heterogeneity in the patterns of collagen synthesis among the substrains could be related to differences in growth rates and the effects of *in vitro* aging (5, 6) on cell phenotype. This would be relevant to the data in Table I, in which the parent (L.T.F.) was PTH responsive and substrains were unresponsive to this hormone. The parent cells had undergone far fewer doublings in comparison to the clones. However, the stability of substrain phenotype with respect to collagen synthesis and PGE<sub>2</sub> response found in these studies and in previous investigations (1–3) argues against aging or senescence as the cause of the phenotypic diversity.

In conclusion, our studies demonstrate that connective tissue cell substrains cloned from normal human dermal tissues are heterogeneous both in terms of synthetic products and in the activity of cell surface receptors for hormones that regulate connective tissue cell function. Selective expansion of cell populations of connective tissue cells *in vivo* could give rise to tissues with aberrant patterns of matrix synthesis. In addition, alterations in responses to hormones that regulate these cells could contribute to the disturbance in normal functional activity. Similar processes may also be involved in connective tissue remodeling under physiologic conditions.

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