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Replication and phenotypic expression of control and scleroderma human fibroblasts: Responses to growth factors

(cell replication/collagen synthesis)

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ABSTRACT **To explore the mechanism of increased collagen synthesis by scleroderma skin fibroblasts *in vitro*, control and scleroderma fibroblasts were compared in confluent monolayer cultures growth-arrested by serum deprivation; responses to optimal mitogenic doses of platelet-derived growth factor, fibroblast growth factor, epidermal growth factor and nerve growth factor were compared. Platelet-derived growth factor had a selective mitogenic effect on control skin fibroblasts not observed with scleroderma skin fibroblasts. None of the factors studied had a selective effect on collagen synthesis independent of cell replication; scleroderma and control fibroblasts responded similarly. Therefore, the growth factors studied may not be involved in generating the activated scleroderma fibroblast directly; platelet-derived growth factor may play an indirect role in fibroblast replication in human fibrotic disorders.**

More than a decade ago, it was observed that fibroblasts from involved skin of patients with scleroderma synthesized increased levels of collagen *in vitro* (1, 2); today the mechanism remains unknown (3–6). During this period, observations of the pathogenesis of scleroderma have focused on vascular and microvascular lesions; on evidence of endothelial damage; on platelet adhesion, aggregation, and release; and on the subsequent activation both of smooth muscle cells to produce the intimal proliferation and of interstitial fibroblasts to produce fibrosis (7–10). A unifying hypothesis would propose the presence of factors released from plasma or platelets which act as stimuli to fibrosis; among such factors, the potent mitogen, platelet-derived growth factor (PDGF), is released during endothelial injury and platelet adhesion to the subendothelium (11). Therefore, the influence of PDGF on skin fibroblast collagen synthesis was studied.

Endothelial damage and platelet interaction with the exposed subendothelium, resulting in release of platelet-derived mitogenic factors (including PDGF), have been proposed, at least indirectly, to play a role in the evolution of the scleroderma vascular lesion. Events preceding endothelial injury are poorly understood; altered immunity leading to humoral or lymphocyte/monocyte mediators of injury has been proposed with supporting evidence (12). If PDGF is the direct mediator of fibroblast activation in scleroderma, the enhanced collagen synthesis of scleroderma fibroblasts should be accompanied by increased cell replication at some stage in the process. Thus, a PDGF mechanism in scleroderma would imply replication of fibroblast populations instead of, or in addition to, the selective stimulation of collagen synthesis per cell; in earlier studies, increased collagen synthesis per cell was observed *in vitro* without evidence of increased cell replication (1, 2). If PDGF is the mediator of fibroblast activation in scleroderma, its action should account for the initial observations of increased collagen synthesis per cell.

A second characteristic of scleroderma fibroblasts in culture has been their insensitivity (of cell replication) to alterations in feeding schedules. This could be interpreted as an insensitivity to PDGF resulting from prior exposure to and maximal stimulation by this serum mitogen *in vivo*. If this were the case, scleroderma fibroblasts should be relatively insensitive to PDGF when compared with control fibroblasts *in vitro*.

In order to examine these possibilities, a systematic study of the effects of PDGF on cell replication and collagen synthesis by control and scleroderma fibroblasts was undertaken. Results demonstrate that PDGF has a selective proliferative effect on control fibroblasts not observed with scleroderma fibroblasts and no selective effect on collagen synthesis. Thus, to be a stimulus for fibroblast activation in scleroderma, PDGF must mediate this activation by its mitogenic action. Similar studies with epidermal growth factor (EGF), fibroblast growth factor (FGF), and nerve growth factor (NGF) are reported herein.

MATERIALS AND METHODS

Skin Biopsies. Two full-thickness skin biopsy specimens (dermis and hypodermis; 4-mm punch) were obtained from the dorsum of the forearm of five scleroderma patients within 3 yr of diagnosis and within 5 yr of the onset of Raynaud's symptoms. All patients had extensive cutaneous and multiple visceral involvement; at the time of biopsy, no patient had organ insufficiency and no patient was receiving medication known to affect connective tissue metabolism. One biopsy was sent for histologic confirmation of scleroderma skin involvement. A healthy donor matched for age, race, and sex of each scleroderma patient underwent a biopsy at an identical site, also histologically confirmed as normal. Each biopsy was placed in medium (containing penicillin and gentamycin) and divided into pieces of 0.5–1 mm^3 each. An X was cut into the bottom of a 60-mm culture dish (Falcon Products, Bio Quest, Cockeysville, NY), the biopsy section was placed at its center, 2 ml of medium [F12K with 15% fetal calf serum (Sterile Systems, Logan, UT) containing penicillin and gentamycin] was carefully added, and the dishes were placed in a 37°C incubator with 5% CO_2/95% air. Medium was replaced daily. Antibiotics were removed after the first passage. After 3–5 days, outgrowth of epithelial cells was seen; fibroblasts appeared beyond the expanding epithelial sheets and around tissue pieces without epithelial outgrowth

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Abbreviations: PDGF, platelet-derived growth factor; NGF, nerve growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; ^{3}H-protein, ^{3}H-labeled total protein.

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at 7–10 days. After 4–5 wk, plates were densely covered with cells of fibroblastic appearance and were subcultured (1, 2).

Reagents. The synthetic medium used in these studies was Coon's modification of Ham's F-12 medium (13, 14) supplemented with sodium ascorbate (50 μg/ml) and Hepes (25 mM) and was prepared as described (15). Amino acids, vitamins, and other organics were purchased from Sigma, Calbiochem, or Schwartz/Mann; Hepes, from Research Organics (Cleveland, OH); trace elements, from Johnson Matthey (United Mineral and Chemical Corporation, New York); and other inorganics, from Baker. The pH was adjusted to 7.6 at room temperature after equilibrating with 5% CO_2/95% air. K-Hanks' medium was made according to Konigsberg's modification of Hanks' balanced salt solution buffered with Hepes at pH 7.4 (16).

Experimental Design. Matched normal and scleroderma skin fibroblasts were harvested from confluent cultures with trypsin between the 4th and 12th passages and were inoculated (2×10^5 cells) in 1.5 ml of F12K medium containing 0.5% fetal calf serum in 35-mm dishes for 24 hr to permit attachment. Triplicate plates were counted and pulsed for 3 hr in serum-free medium with [^{3}H]proline (for collagen and total protein assay) or with [^{3}H]thymidine (to measure proliferation) to demonstrate that cells were synthesizing collagen and replicating before beginning each experiment. Experiments were discarded if cell death or contamination was detected by low incorporation of isotope.

After attachment for 24 hr, medium was changed to one of the following: F12K with 15% fetal calf serum (F12K/15 medium), F12K with 0.5% fetal calf serum (F12K/0.5 medium), or F12K with 0.5% fetal calf serum and PDGF (F12K/0.5 medium containing PDGF) or other growth factors in optimal dose. After a 72-hr incubation, cells were incubated with isotope and assayed for collagen synthesis, protein synthesis, thymidine incorporation, and cell number.

Cell Density Optimum and PDGF Dose Response Curve. Normal fibroblasts at the fourth passage were placed in quadruplicate 35-mm dishes in F12K/15 medium in cell densities of 0.5, 1.0, 2.0, and 4.0×10^5 cells per dish. At 24 hr, medium was changed to F12K/0.5, with alternate plates of each cell density receiving PDGF (Collaborative Research, Waltham, MA) at a previously determined optimal dose of 3 proliferative units per ml (determined by maximal [^{3}H]thymidine incorporation). After an additional 24 hr, medium was changed to F12K without fetal calf serum, and half of the plates were pulsed with 15 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]proline (New England Nuclear) for 3 hr. The cells were scraped from the plates with a rubber policeman and assayed for collagen synthesis. The remaining plates were pulsed with [^{3}H]thymidine (3 μCi for 3 hr) and harvested with 0.1% trypsin and K-Hanks' medium; cell counts were made, and the cells were harvested onto glass fiber filters, placed into scintillation fluid, and assayed for radioactivity. For collagen synthesis (Fig. 1), cell proliferation, and [^{3}H]thymidine incorporation (data not shown), stimulation with PDGF was similar at all cell densities studied; in all subsequent experiments, an initial cell density of 2×10^5 was used (2×10^4 cells per cm^2 surface area).

By using optimal cell densities as determined above, a dose–response curve for PDGF was determined with control fibroblasts. After 24 hr in F12K/15, the medium was again changed to F12K/0.5 medium containing PDGF in the following concentrations: 1.5, 3.0, 6.0, 12.0, and 15.0 units per dish. [Purified PDGF at 1 ng/ml (0.1 nM) stimulates replicative DNA synthesis and cell proliferation in quiescent, density-arrested cultures of BALB/c 3T3 cells; a unit of PDGF activity is defined as the amount that induces 50% of the above cells (approximately 10^4) to synthesize DNA; see ref. 17]. After 72 hr, cells were incubated with isotope, harvested, and assayed. A peak of cell counts (Fig. 2), collagen synthesis, and [^{3}H]thymidine uptake (data not shown) was reached at 3 units of PDGF per ml. All subsequent experiments were done with

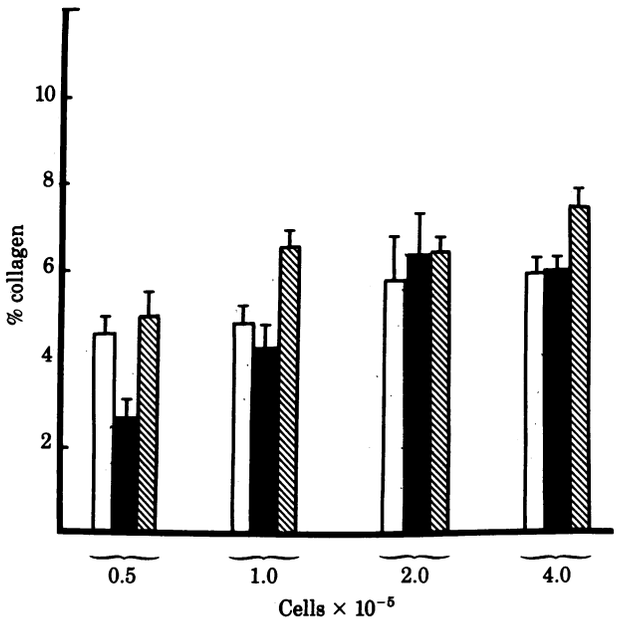

FIG. 1. Relationship of collagen synthesis to cell density by control skin fibroblasts in media supplemented with PDGF (□), high (15%) fetal calf serum (■), and low (0.5%) fetal calf serum (▨). At the higher cell densities, which are near confluence, a plateau of synthesis in PDGF-supplemented cultures was observed. Data are presented as percentage collagen ([^{3}H]proline incorporated into collagen/[^{3}H]proline incorporated into total protein, with correction for the abundance of proline/hydroxyproline in collagen).

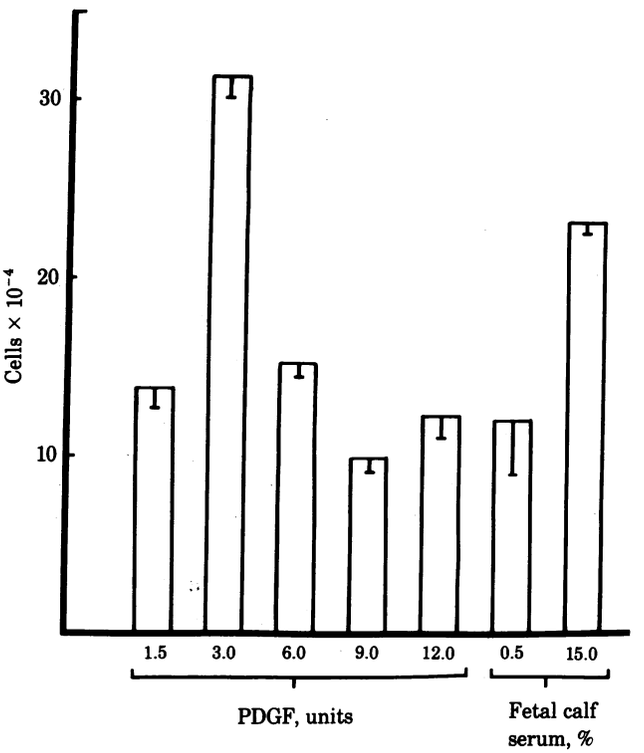

FIG. 2. Dose–response curve for PDGF with control skin fibroblasts as determined by cell counts. Data are means ± SEM of triplicate plates.

PDGF at 3 units/ml, which corresponds to the optimal proliferative dose in the supplier's hands, using 3T3 cells at confluence. Similar experiments with a scleroderma cell line indicated the same optimal cell density and optimal PDGF concentration. Experiments with FGF, EFG, and NGF (Collaborative Research, Waltham, MA) were performed using concentrations optimal for proliferation as determined by similar techniques.

Collagen Assay. The method used was a modification of the collagenase procedure described by Peterkofsky and Diegelmann (18). Cells were exposed to [^{3}H]proline and harvested, protein from cells and media were precipitated together with trichloroacetic acid and redissolved in 0.2 M NaOH, and duplicate aliquots were incubated with no enzyme (for spontaneously released ^{3}H), protease-free collagenase (for [^{3}H]collagen), or Pronase [for ^{3}H-labeled total protein (^{3}H-protein)]. Digestions were stopped by trichloroacetic acid precipitation, and supernatants were assayed by scintillation spectrometry. Collagen and total protein synthesis were estimated by subtracting spontaneously-released ^{3}H dpm from collagenase-released ^{3}H dpm and from Pronase-released ^{3}H dpm, respectively; an empirical formula was used to correlate data from the modified procedure (use of Pronase) with those produced by the original method; technical details and validity of the modification have been published (19).

RESULTS

Cell Viability. Preliminary experiments to compare viability and growth characteristics of the cell strains revealed no differences in cell loss between control and scleroderma cells of the same pair after initial trypsinization and resuspension (2×10^4 cells per cm^2 in F12K/0.5 medium); different pairs studied at different times showed losses between 15% and 50% of the cells planted; neonatal foreskin fibroblasts tested similarly showed no loss. At 24 hr, cell counts revealed losses of 30–49%, with no differences between control and scleroderma partners of the same pair. At 72 hr, control cells in F12K/0.5 medium were reduced by 0–30%; scleroderma cells remained unchanged from 24-hr cell counts. Initial losses were due to cells failing to adhere to the coated plastic surface. These data further demonstrate the relative stability of scleroderma fibroblasts *in vitro* and the difficulty in maintaining control adult human fibroblasts at the low serum concentrations necessary to study mitogenic factors.

Replicative responses to growth factors are shown in Table 1. Results are presented as percentages of low-serum controls to permit comparisons between different experiments. PDGF stimulated control skin fibroblasts more than it did scleroderma fibroblasts and more than other growth factors or serum stimulated control fibroblasts. Scleroderma cells were insensitive to added PDGF, suggesting that they may have been exposed to this mitogen *in vivo*. In this experimental setting, FGF, EGF, and NGF, which were mitogenic for human newborn foreskin fibroblasts, did not stimulate either control or scleroderma adult skin fibroblasts to replicate, suggesting that mature fibroblasts may lose their capacity to respond to these specialized growth factors.

Table 1. Replicative responses to growth factors

Growth factors	Fibroblast response, % of low-serum control*	
	Skin control	Scleroderma
FGF	99 ± 6	106 ± 3
EGF	109 ± 4	94 ± 5
NGF	101 ± 10	103 ± 13
PDGF	160 ± 4	110 ± 3
Fetal calf serum, 15%	118 ± 5	101 ± 5

* Expressed as percentage of the response on growth-arresting low-serum control plates (0.5% fetal calf serum), ± SEM.

Figs. 3 *A* and *B* present a representative experiment comparing collagen and protein synthesis in F12K/15 medium, F12K/0.5 medium, and F12K/0.5 medium with PDGF by control and scleroderma fibroblasts. In F12K/0.5 medium containing PDGF, levels of collagen and protein synthesis were consistently below those in F12K/15 medium, suggesting that PDGF is not a selective stimulus for collagen or protein synthesis in this system (see Table 1). Also, there was no detectable difference between the effect of PDGF on control or scleroderma fibroblasts, suggesting that prior exposure to PDGF is not the basis for increased collagen synthesis by scleroderma fibroblasts. Thus, the precise stimulus or stimuli for fibroblast activation in scleroderma remains unknown. When normal-scleroderma pairs were compared, dpm of [^{3}H]collagen per cell and percentage collagen per cell were greater in scleroderma cells in all conditions tested ($P < 0.001$; Fig. 3*A*), an observation repeatedly confirmed in the literature (1, 2, 4–6).

Similar studies were conducted with FGF, EGF, and NGF. As shown in Table 2, growth factors in the presence of low serum (F12K/0.5 medium) did not selectively stimulate either collagen or protein synthesis when corrected for cell replication and compared with the effect of F12K/15 (high serum) or F12K/0.5 (low serum). Moreover, in no case except NGF did growth factors (in low serum) equal or exceed the stimulation observed with high serum. Results with NGF seemed to show equal or slightly greater stimulation with scleroderma than with control

Table 2. Effects of growth factors on collagen and protein synthesis by normal and scleroderma fibroblasts

Growth factor serum level	Synthesis per fibroblast*	
	Normal	Scleroderma
	Collagen	
FGF High	0.56 ± 0.2	0.31 ± 0.03
FGF Low	0.77 ± 0.17	0.42 ± 0.04
EGF High	0.37 ± 0.16	0.46 ± 0.05
EGF Low	0.71 ± 0.1	0.63 ± 0.06
NGF High	0.47 ± 0.02	0.45 ± 0.04
NGF Low	0.75 ± 0.12	1.19 ± 0.31
PDGF High	0.47 ± 0.07	0.7 ± 0.07
PDGF Low	1.07 ± 0.26	0.95 ± 0.08
	Protein	
FGF High	0.77 ± 0.18	0.4 ± 0.02
FGF Low	0.91 ± 0.1	0.6 ± 0.04
EGF High	0.55 ± 0.03	0.57 ± 0.04
EGF Low	0.76 ± 0.1	0.86 ± 0.7
NGF High	0.5 ± 0.06	0.62 ± 0.02
NGF Low	0.88 ± 0.05	1.17 ± 0.29
PDGF High	0.6 ± 0.09	0.69 ± 0.09
PDGF Low	0.83 ± 0.14	1.03 ± 0.14

*Expressed as ratio of the value for F12K/15 (high serum) or F12K/0.5 (low serum) to the value in low serum (F12K/0.5) corrected for cell replication. Data are means of triplicate plates of two control/scleroderma pairs ± SEM.

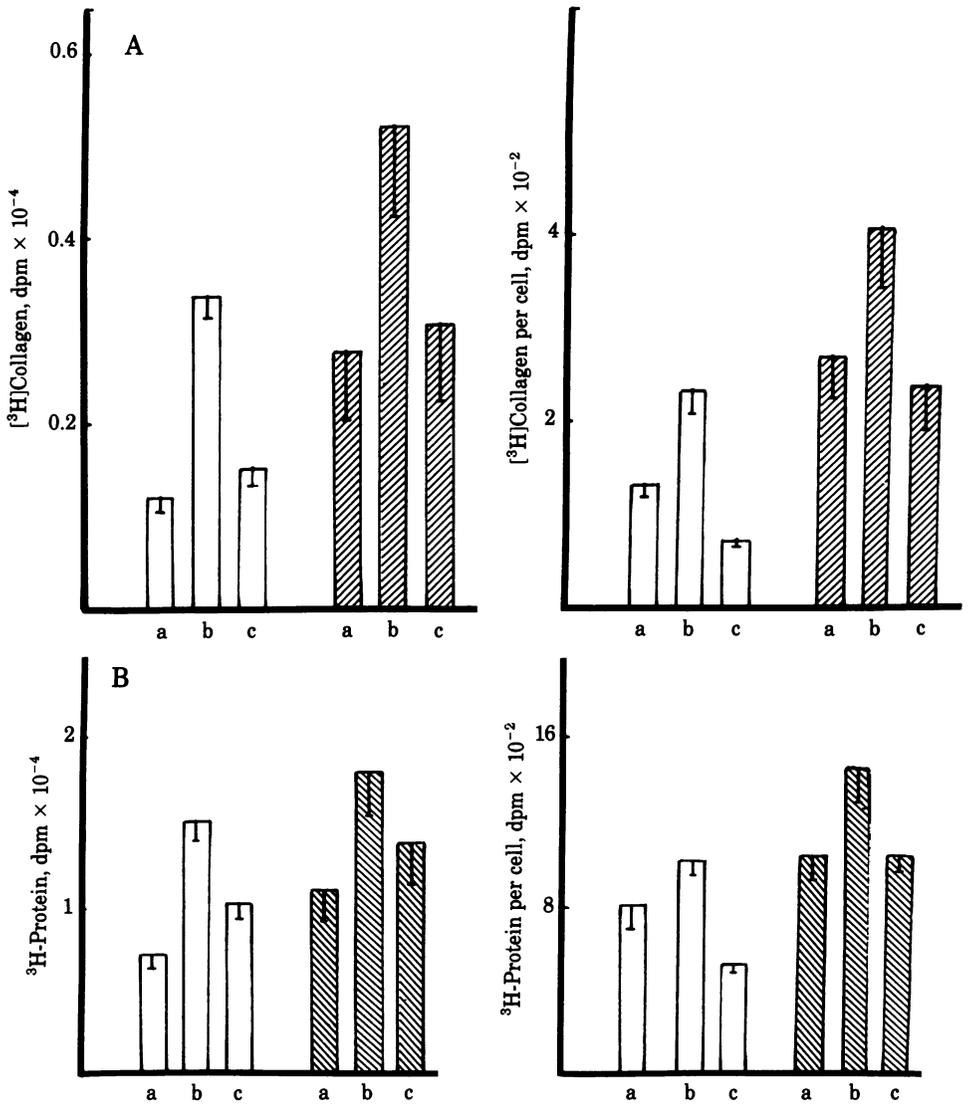

FIG. 3. Collagen (*A*) and protein (*B*) synthesis in control (□) and scleroderma (▨) fibroblasts in response to low serum (F12K/0.5 medium; columns a), high serum (F12K/15 medium; columns b) and low serum with growth factor (F12K/0.5 medium with 3 units of PDGF per ml; columns c). PDGF did not substitute for serum and does not distinguish control from scleroderma fibroblast responses. (*A*) Data are expressed both as dpm of [^{3}H]collagen and as dpm of [^{3}H]collagen per cell. (*B*) Data are expressed both as dpm of ^{3}H-protein and as dpm of ^{3}H-protein per cell.

(normal) cells; wide variability (SEM, 0.3) warrants further study.

DISCUSSION

The mechanism by which collagen is synthesized and deposited in increased quantity in scleroderma and in other human fibrotic diseases is unknown. Morphologically there is close association between chronic inflammatory cells and fibrosis. In scleroderma, perivascular mononuclear cell accumulation is recognized as a prefibrotic manifestation of this multistage disorder. It has been demonstrated that the activation of immune networks, either by selective antigen stimulation (20) or by nonspecific mitogens (21, 22), leads to the elaboration of soluble factors, still poorly identified, which modulate fibroblast proliferation and collagen synthesis. The precise role of these immune events in the pathogenesis of scleroderma awaits further investigation.

Associated with this early monocyte accumulation is a distinctive injury of the vascular endothelium that may be mediated by a serum activity selectively cytotoxic to endothelial cells *in vitro* (23, 24). A damaged endothelium allows release of vascular-derived factors to stimulate interstitial fibroblasts to replicate, synthesize collagen, and produce fibrosis. The present work explores the effects of the powerful serum mitogen for smooth muscle cells and fibroblasts, PDGF, which is released from the platelet α granule on the interaction of the blood platelet with the subendothelium of a damaged blood vessel. Such interaction involves adhesion, aggregation, and release of platelet granule constituents (25). Under the conditions of the present study, PDGF was selectively mitogenic for control fibroblasts, was not mitogenic for scleroderma fibroblasts, and did not selectively stimulate fibroblast collagen or protein synthesis.

Central to defining the precise mechanism of fibrosis in scleroderma is an understanding of the role of proliferation or replication of fibroblasts in this and other human fibrotic disorders. Directed migration by fibroblast chemotaxis also must be considered (26). Proliferation of fibroblasts may contribute to fibrosis both by increased number of cells—all producing equivalent amounts of connective tissue—and by the selection of fibroblasts after multiple population doublings, which inherently produce large quantities of collagen (27). The process of fibroblast replication has been viewed as differentiation (28).

Most *in vitro* studies of fibroblast behavior *vis-a-vis* fibrosis have focused on collagen or proteoglycan synthesis and have not assessed the relationship of cell replication to the synthesis and assembly of extracellular matrix. Whether a specific linkage exists between fibroblast replication and phenotypic expression (collagen synthesis)—for example, in lymphocyte subsets, where replication and lymphokine production are closely associated (29)—remains unknown. PDGF has been shown to be a necessary but not sufficient mitogen for fibroblasts *in vitro* and shares a cooperative role with somatomedins for full mitogenic expression (17). In the present study, no evidence could be found for a selective role of PDGF on collagen synthesis in control or scleroderma fibroblasts. Nonetheless, the selective mitogenic effect of PDGF on control fibroblasts and the insensitivity to PDGF of fibroblasts from scleroderma subjects suggests that scleroderma fibroblasts may have been altered by PDGF exposure *in vivo*.

The growth factors reported in this study showed no effect on collagen synthesis over and above their effect on fibroblast replication. In this regard a caveat is suggested: for any biological or pharmacologic agent to be shown to have a direct effect on fibroblast collagen synthesis, it should be clearly demonstrated that the observed effect is not produced by mitogenesis. This caveat should apply to matrix components (NH_2-terminal procollagen peptides), to sympathetic neurotransmitters (β-adrenergic agonists and antagonists), to inflammatory mediators (prostaglandins, serotonin, histamine), or to drugs (bleomycin). A possible common effector for all of these agents could be cyclic AMP, whose effect on collagen synthesis may be secondary to a replicative effect. A large number of studies (1, 2, 30) have not been well controlled for effects on cell replication. The use of cell counts, cell DNA, or cell protein as denominators may not reflect mitogenic signals unless sufficient time elapses for completed cell replication.

The regulation of fibrosis and collagen synthesis in developmental and pathological settings is not understood. In pathologic states, fibrosis often follows inflammatory and granulomatous responses. The increasing knowledge of the role of the blood platelet in inflammation led us to study PDGF as a possible stimulus of fibrosis in scleroderma. Scleroderma fibroblasts demonstrated an insensitivity to PDGF in these studies. Further study is needed to determine whether inflammation generates specific signals that stimulate collagen synthesis in resident fibroblasts or whether fibroblast populations are attracted to or selected by replication for the initiation of fibrosis.

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