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Stimulation of *in vitro* human skin collagenase expression by platelet-derived growth factor

(collagenase activity/immunoreactive protein/biosynthesis/cell-free translation)

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ABSTRACT **Platelet-derived growth factor (PDGF) is both chemoattractant and mitogenic for stromal cells. Here, we examined the effects of PDGF on collagenase expression by normal human skin fibroblasts. Culturing cells for 24 hr in the presence of PDGF at 0–180 ng/ml resulted in a dose-dependent, saturable increase in collagenase activity in the culture medium that was paralleled by equal increases in immunoreactive collagenase protein, suggesting enhanced synthesis of a catalytically unaltered enzyme. The specificity of this effect was demonstrated by comparing the collagenase-stimulatory effect with that on total protein synthesis and DNA synthesis. Under *in vitro* conditions that produced a 2.5-fold increase in collagenase synthesis, there was an ≈20% increase in total protein synthesis and no change in DNA synthesis. In addition, platelet factor 4, another platelet-derived protein, caused a <20% increase in collagenase expression. In time-course studies, stimulation of collagenase synthesis was first observed 8–10 hr after exposure to the growth factor. Conversely, when cells were primed with PDGF for ≈24 hr and the stimulator was then removed, an increased rate of synthesis was seen for an additional ≈6 hr, after which the rate reverted to control levels. Since the kinetic data suggested a possible pretranslational effect, fibroblasts cultured with PDGF were used to prepare mRNA. In cell-free translation, total protein synthesis was essentially unaltered; however, the growth factor caused a >2-fold increase in translatable collagenase mRNA. The data suggest that PDGF specifically modulates collagenase synthesis, possibly through a series of events that lead to increased transcription or preferential translation of collagenase mRNA.**

A number of peptide growth factors are important in mediating cellular proliferation, tissue differentiation, and/or tissue repair. Among these, platelet-derived growth factor (PDGF) is of importance because of its putative role in initiating a series of events in cells that govern cell replication in such seemingly diverse processes as repair of blood vessel injury and atherogenesis (1), wound healing in physiologic circumstances (2, 3), and possibly—by its homology to the putative transforming protein of the simian sarcoma virus, $p28^{v\text{-}sis}$—in the molecular events that govern malignant transformation (4–8).

PDGF is postulated to function as a mitogen in wound healing when blood vessel integrity is compromised and platelet activation occurs (2, 3). Smooth muscle cells and fibroblasts as well as inflammatory cells are strongly attracted by low concentrations (10–20 ng/ml) of PDGF (9–12). Several of the events of wound healing—fibrin formation, fibroblast influx, collagen synthesis, and connective tissue reorganization to yield a healed wound of high tensile strength—may be temporally correlated with the release of PDGF.

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Because of the profound chemoattractant and mitogenic effect of PDGF on mesenchymal cells—specifically fibroblasts—we have postulated that this protein might influence certain biochemical events in such cells. In this regard, skin fibroblasts synthesize, as one of their principal gene products, collagenase (13). This enzyme functions at the rate-limiting step in initiating collagen degradation (14). In addition, collagenases have been shown to play a role in the physiologic restructuring of collagen during wound healing (15–18) and in the exaggerated cutaneous connective tissue destruction that characterizes the hereditary blistering disorder, recessive dystrophic epidermolysis bullosa (19–21).

In this paper we have used PDGF as a probe to gain insight into the action of this agent on human skin fibroblasts. Our aims were threefold: (*i*) to determine whether PDGF modulates collagenase expression by human skin fibroblasts, (*ii*) to determine the specificity of this putative effect, and (*iii*) to assign a biochemical mechanism of action for the putative PDGF effect at the cellular level.

METHODS

Preparation of Platelet Factors. Electrophoretically homogeneous PDGF I and II were prepared as described (22). Briefly, platelet-rich plasma was subjected sequentially to Sulfadex ion-exchange chromatography, heat inactivation, CM-Sephadex ion-exchange chromatography, and Blue-Sepharose chromatography, followed by gel filtration chromatography. This 100,000-fold purified material was >95% homogeneous on gel electrophoresis.

Purified platelet factor 4 (PF 4) was obtained as detailed earlier (23). Purification was accomplished by ammonium sulfate precipitation, heparin affinity chromatography, and gel filtration. The purified protein was a single band on 15% polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

Both PDGF and PF 4 were stored at −70°C until used. Immediately prior to the addition to human skin fibroblast target cells, the factors were diluted to the desired final concentration in Dulbecco's modified Eagle's medium (DME medium) containing 1% (wt/vol) human albumin and antibiotics (DME medium/albumin).

Cultures of Human Skin Fibroblasts. Normal skin fibroblasts were cultured for use as target cells for testing effects of the PDGF. These fibroblasts were derived from the skin of six different individuals of both sexes ranging in age from 8 to 40 yr. All cells were passed sequentially in a 1:4 ratio for growth area and were used in passages 6–11. Briefly, cells were grown in plastic culture flasks (Corning) in DME medium containing 0.03 M Hepes buffer (pH 7.6), 10% fetal calf serum, and 200 units of penicillin and 200 μg of streptomycin per ml at 37°C (24). In most of the experiments

Abbreviations: PDGF, platelet-derived growth factor; PF 4, platelet factor 4.

to determine the concentration of collagenase in the culture medium, fibroblasts were grown to early confluence, a cell density at which collagenase expression is greatest (25). Prior to testing the effects of PDGF, culture medium containing 10% fetal calf serum was removed and replaced with DME medium containing 2% fetal calf serum for 48 hr to deplete any stores of PDGF in the serum. Serum-containing medium was then removed, the cells were washed four times with prewarmed DME medium, and the cultures were maintained in DME medium/albumin in the presence or absence of the PDGF for up to 24 hr. Under these conditions, cell proliferation was not observed. After the incubation, the DME medium/albumin was removed, made 0.05 M in Tris·HCl (pH 7.5) and 0.01 M in $CaCl_2$ and stored at −20°C for enzyme and/or immunologic assay (see below).

Collagenase Activity. Human skin procollagenase was activated proteolytically with trypsin as described (24). For each enzyme preparation, a range of trypsin concentrations (0.2–5.0 μg of trypsin per 100 μl of enzyme sample) was used to ensure that maximal collagenase activity was measured (24). After preincubation with trypsin for 10 min at 25°C, at least a 5-fold molar excess of soybean trypsin inhibitor was added to inhibit further trypsin activity. Each mixture was then assayed for collagenase activity at 37°C in 0.05 M Tris·HCl, pH 7.5/10 mM $CaCl_2$ with native, reconstituted [^{14}C]glycine-labeled collagen fibrils containing ≈5000 cpm per substrate gel (26). All assays were linear with time and protein concentration.

Immunoassay of Collagenase. To measure immunoreactive collagenase in the DME medium/albumin portions were allowed to react in the ELISA for human skin collagenase as detailed (27). Human skin procollagenase for use as the immunogen, for coating the ELISA plates, and for developing the standard curve was purified to homogeneity from the medium of human skin fibroblast cultures as described by Stricklin *et al.* (28). This preparation was used to prepare functionally specific antiserum to the enzyme as given in detail (29). The antiserum gave a single immunoprecipitin band when allowed to react in Ouchterlony analysis with either the crude culture medium or with the antigen that had been purified to homogeneity. Furthermore, a gamma-globulin fraction of this antiserum produced >90% inhibition of collagenase activity compared to <5% inhibition by a nonimmune gamma-globulin preparation (29).

Biosynthesis of Collagenase. In order to examine the effects of PDGF on the biosynthesis of skin collagenase, cultures were incubated in leucine-free DME medium containing antibiotics and 30 mM Hepes buffer (13). [^{3}H]Leucine was added to 10–50 μCi/ml (1 Ci = 37 GBq). After the desired incubation period, the medium was harvested and dialyzed at 4°C against several changes of 0.05 M Tris·HCl, pH 7.5/0.15 M NaCl (Tris/NaCl buffer) prior to immunoprecipitation. The cell layer was washed three times with Hank's balanced salt solution at 4°C, lysed by sonication, and assayed for total protein (30). Labeled human skin collagenase was precipitated from the medium with specific antiserum to the enzyme (29). A typical reaction mixture contained 50–200 μl of the ^{3}H-labeled culture medium, 3–10 μl of rabbit anti-human skin collagenase serum, and a sufficient quantity of electrophoretically pure carrier human skin collagenase to give precipitation in the zone of equivalence (13). The resultant precipitates were harvested by centrifugation, washed four times with Tris/NaCl buffer, and were dissolved in sample buffer for gel electrophoresis (see below) or in ACS scintillant solution for counting. As a control for background radioactivity caused by nonspecific trapping of labeled proteins in the immunoprecipitate, the same volume of labeled medium was incubated with 3–10 μl of antiserum to ovalbumin and a sufficient amount of ovalbumin shown by previous titration to give an equivalence-point precipitation in this system (13). The amounts of protein immunologically precipitated in the ovalbumin–anti-ovalbumin and collagenase–anti-collagenase systems were equal.

Translation of Collagenase mRNA. The effects of PDGF also were assessed in cell-free translation by using the rabbit reticulocyte lysate system as described (31). In each experiment we utilized one large roller bottle (1585 cm^2) of confluent human skin fibroblasts for harvesting mRNA by the method of Rowe *et al.* (32). The control cells were cultured in the absence of PDGF for 24 hr, while the stimulated cultures were maintained for the same time period in the presence of PDGF. At the end of this time, culture medium was harvested for quantitation of collagenase activity, and the cells were harvested for preparation of mRNA. After disruption of the cells (32), nucleic acids were extracted with phenol and chloroform followed by several precipitations with ethanol, and mRNA was then further purified on a column of oligo(dT)-cellulose. The oligo(dT)-cellulose column eluate was precipitated with 0.1 M NaCl/66% ethanol at −20°C, and the resultant mRNA was dissolved in water and stored at −70°C (31). For translation, optimum concentrations for the various reagents per ml of final volume were 0.4 ml of lysate, 8–20 μg of mRNA, 0.1–1.0 mCi of [^{35}S]methionine, 80 mM potassium acetate, 1 mM magnesium acetate, 0.75 mM spermidine, 20 μg of transfer RNA, 0.2 mM ATP, 8 mM creatinine phosphate, 1 mM dithiothreitol, and 20 mM Hepes buffer (pH 7.5). After translation, protein synthesis was stopped with 1/10 vol of ribonuclease A (1 mg/ml) and bovine serum albumin was added to a final concentration of 0.2%. The volume was brought to 2 ml with Tris/NaCl/Triton/EDTA buffer, and the samples were ultracentrifuged at 35,000 rpm (31). The postribosomal supernatant was made 10 mM with unlabeled methionine prior to precipitation of the translation products with trichloracetic acid or anti-human skin collagenase antiserum. To minimize nonspecific trapping of radioactive material, immunoprecipitation mixtures were layered over discontinuous sucrose gradients and centrifuged for 15 min at 12,000 × *g* at 4°C (31). The precipitates were dissolved for assay in ACS scintillant solution. Comparisons of translational activity between control and stimulated mRNA preparations were carried out simultaneously.

Analytical NaDodSO$_4$ Gel Electrophoresis. In order to assess the qualitative nature of the immunoprecipitated labeled enzyme protein, the washed immunoprecipitates were dissolved in 25–100 μl of sample buffer containing 0.065 M Tris·HCl (pH 6.8), 8 M urea, 3% NaDodSO$_4$, and 2% 2-mercaptoethanol for gel electrophoresis. The precipitates were dissolved in a boiling water bath for 5 min prior to electrophoresis, one drop of glycerol containing bromphenol blue was added, and the samples were applied to a discontinuous NaDodSO$_4$ slab gel made with 10% (wt/vol) acrylamide and 0.27% *N,N'*-methylenebisacrylamide in the separating gel (33). Electrophoresis was carried out at 70 mA per mm of slab thickness. For fluorography, the slab gels were equilibrated with dimethyl sulfoxide, immersed in 20%, 2,5-diphenyloxazole in dimethyl sulfoxide for 3 hr, rinsed in distilled water for 20 hr, and dried under vacuum. The dried gels were exposed to Kodak XR-5 X-Omat R film (Eastman Kodak) at −70°C (34). Densitometric scans of the fluorographs were done with a Zeiss PM6 spectrophotometer.

DNA Synthesis. Mitogenic activity was measured by the incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material. Briefly, fibroblasts preincubated in DME medium containing 2% fetal calf serum for 48 hr were washed and exposed to DME medium/albumin in the presence or absence of PDGF for the same 24-hr periods used to determine collagenase expression. Cells were then washed with Hank's balanced salt solution and fixed with 5% trichloroacetic acid on ice prior to determining the radioactivity.

RESULTS

Initially we examined the effect of various concentrations of PDGF on collagenase expression, measured as immunoreactive protein secreted by human skin fibroblasts in culture (Fig. 1). The response of these cells was dependent on the concentration of PDGF added to the medium. Half-maximal stimulation by PDGF of collagenase expression occurred at 20–40 ng/ml; maximum secretion was observed at 90–120 ng/ml.

We also determined the effect of PDGF on collagenase activity in human skin fibroblast cultures. As shown in Table 1, addition of PDGF to the culture medium resulted in an ≈2-fold increase in trypsin-activatable collagenase activity. A parallel increase was observed in immunoreactive protein released into the culture medium, suggesting that the stimulation of collagenase activity was due to increased synthesis. The collagenase that was synthesized under the stimulation of the PDGF appeared to be catalytically equivalent to that found in control cultures, since the activity per immunoreactive protein was essentially unchanged in the stimulated cultures (Table 1). Direct addition of PDGF to the collagen/collagenase reaction mix *in vitro* did not alter the catalysis (data not shown).

In order to gain insight into the mechanisms involved in the response of skin fibroblasts to PDGF, we examined the kinetics of stimulation of collagenase expression by the cytokine (Fig. 2). For these experiments, fibroblasts were placed in DME medium/albumin in the presence or absence of PDGF, and portions of the culture medium were harvested at specific time intervals for measurement of immunoreactive collagenase protein released into the culture medium. Accumulation of collagenase protein in the non-PDGF-stimulated cultures was linear for up to 24 hr, the longest time point examined in these experiments. In the PDGF-stimulated cultures, the accumulation of collagenase was equal to that seen in the nonstimulated cultures for approximately the first 8–10 hr, after which the stimulation by PDGF resulted in a new rate of accumulation. In the nonstimulated cultures, the rate of accumulation of collagenase protein in the culture medium was ≈0.26 μg/mg of cell protein per hr. After stimulation, the rate of collagenase accumulation was 0.48 μg/mg of cell protein per hr (Fig. 2*A*).

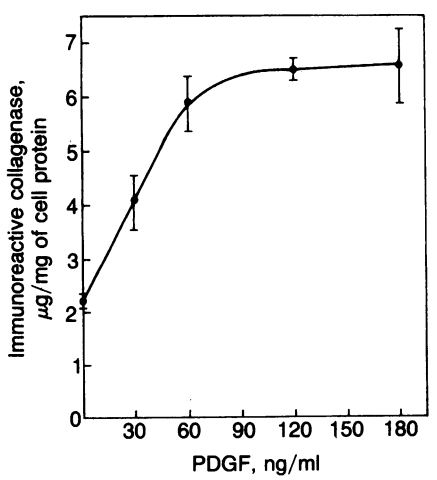

FIG. 1. Effect of PDGF concentration on collagenase expression in human skin fibroblast cultures. Cells were placed in DME medium/albumin in the presence of the indicated concentrations of PDGF for 24 hr. Immunoreactive collagenase was determined by ELISA. Cells were harvested for protein. Data are presented as means ± SEM of μg of collagenase per mg of cell protein in duplicate cultures.

Table 1. Catalytic efficiency of human skin collagenase

Culture conditions	Collagenase activity, cpm/ml	Immunoreactive collagenase, ng/ml	A/I,* cpm/ng
Control	10,650 ± 1,546	1615 ± 112	6.6
PDGF	19,842 ± 1,518	3177 ± 546	6.3

In four separate experiments, fibroblasts were cultured in the presence of 100–180 ng of PDGF per ml for comparison with activity and immunoreactive protein in the medium of control cultures containing no PDGF. The data are expressed as means ± SEM.
*A/I, activity per immunoreactive protein.

We also determined the accumulation of immunoreactive collagenase protein in stimulated or nonstimulated cultures after withdrawal of the PDGF from the culture medium (Fig. 2*B*). For these release experiments, cell cultures that had been primed with the PDGF for 24 hr were depleted of culture medium, washed briefly with prewarmed DME medium/albumin, and then maintained in the same medium for an additional 24 hr. In the unstimulated cultures, accumulation of collagenase protein was again linear at a rate of 0.24 μg/mg of cell protein per hr for the entire experiment. In contrast, the rate of accumulation of collagenase protein in the culture medium in the previously stimulated cultures was 2.5–3 times greater (0.67 μg/mg of cell protein per hr) for the first 4–6 hr, after which the rate of accumulation of enzyme protein in the culture medium returned to levels seen in the nonstimulated cultures. Thus, the kinetics of release from the effects of the PDGF were the reciprocal of those of its stimulatory action, except that the latent period for stimulation was approximately twice as long.

We next turned our attention to biosynthetic studies of collagenase. Fibroblasts were placed in DME medium/albumin in the presence or absence of PDGF and [^{3}H]leucine. As shown by the densitometric scan of the specifically immunoprecipitated collagenase protein in Fig. 3, the addition of PDGF to the culture medium resulted in a quantitative increase in newly synthesized enzyme protein. In a second series of experiments, we explored the specificity of the effect by comparing the action of PDGF and another purified platelet α-granule protein, PF 4, on collagenase synthesis, total protein synthesis, and DNA synthesis (Table 2). Under essentially serum-free *in vitro* conditions, PDGF produced a 2- to 3-fold increase in collagenase synthesis, while there was no stimulation of DNA synthesis and only a 20% increase in total protein synthesis. In contrast, PF 4 produced only a

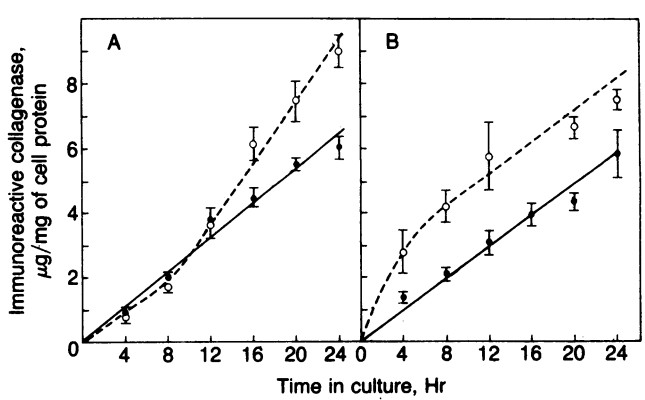

FIG. 2. Kinetics of the onset (*A*) and release (*B*) of collagenase expression in human skin fibroblast cultures in response to PDGF. Cells were maintained in DME medium/albumin in the absence (●—●) or presence (○---○) of 100 ng of PDGF per ml. At the indicated times, portions of medium from duplicate cultures were harvested for determination of immunoreactive collagenase. The data are expressed as means ± SEM of μg of collagenase per mg of cell protein.

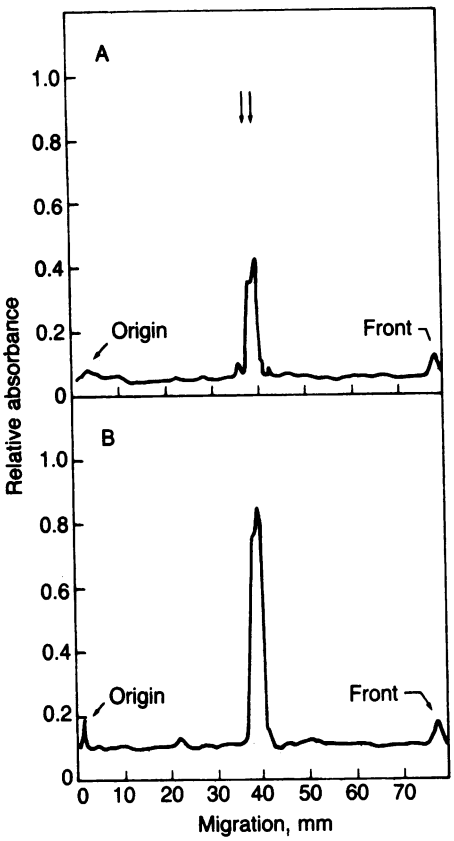

FIG. 3. Effect of PDGF on the biosynthesis of human skin collagenase: densitometric scan of procollagenase immunoprecipitated from culture medium of fibroblasts maintained for ≈24 hr in the absence (*A*) or presence (*B*) of 100 ng of PDGF per ml. [^{3}H]Procollagenase was precipitated from crude culture medium and subjected to electrophoresis on a NaDodSO$_4$/polyacrylamide gel. After fluorography, a densitometric scan was made as described. The arrows indicate the electrophoretic positions of the authentic ≈60- and ≈55-kDa procollagenase species. The cell protein content of the cultures was 0.23–0.25 mg.

slight stimulation in collagenase synthesis (Table 2). At higher concentrations of PF 4 (up to 2500 ng/ml), no further stimulation of collagenase synthesis was observed (data not shown).

Because the kinetic data (Fig. 2) suggested the possibility of stimulation of collagenase synthesis at a pretranslational level, we used cells maintained in the presence or absence of PDGF to prepare mRNA for translation in the rabbit reticulocyte lysate system. Fibroblasts grown in roller bottles were placed in the appropriate medium for ≈22 hr. At the end of that time, the culture medium was harvested for measurement of trypsin-activatable collagenase activity and immunoreactive collagenase in the culture medium as indices of the degree of stimulation in the intact cells. After this, the cells were harvested for processing of mRNA for use in cell-free translation. As shown in Table 3, intact cells secreted a 2- to 3-fold increase of collagenase into the culture medium. Similarly, in the cell-free translation, the percentage of protein synthesis devoted to collagenase synthesis increased ≈2-fold (i.e., from 0.4% to 1.0%). In contrast, there was little (≈10%) stimulation of total translational activity directed by the mRNA harvested from the PDGF-stimulated cultures.

DISCUSSION

The skin is composed principally of two types of tissue, the epidermis and the dermis. After wounding, repair of these two tissues follows a morphologically dynamic pattern in which wound closure and dermal repair form critical first steps upon which depend epidermal repair and redifferentiation. Cellular migration and division must occur within the dermis as initial events. Almost simultaneously collagen is laid down, but to provide a wound of acceptable tensile strength, restructuring of this collagen must occur (15). In this regard, collagenases appear to play a critical role—one in which their maximal expression can be correlated temporally with a period of reorganization of collagen (15–18, 35, 36).

That PDGF functions in initiating directed cell migration and division is accepted (1). However, the capacity to respond to PDGF by increasing expression of a secretory gene product required for remodeling—such as collagenase—would represent an efficient mechanism for controlling molecular events by the organism.

The present study indicates by several different criteria that the increase in collagenase activity brought about by PDGF is due to stimulation of enzyme synthesis. By using both a specific ELISA for collagenase and immunoprecipitation of biosynthetically labeled collagenase, increased enzyme protein was made in response to PDGF. The kinetics of the response to PDGF suggest a complex series of events and possibly a pretranslational level of action, a postulate made more likely by the observation of an increase in translatable collagenase mRNA equal to the degree of stimulation observed in intact cells (Table 3). Whether this increase occurs as part of the function of PDGF as a competence factor is unknown. Nevertheless, it seems reasonable to hypothesize that part of the response to PDGF that involves induction of mRNA and is temporally correlated with the synthesis of certain cytoplasmic proteins (37, 38) may in-

Table 2. Specificity of the effect of PDGF on collagenase expression in human skin fibroblast cultures

Culture conditions	Immunoreactive collagenase, μg/mg of cell protein	% C	Total protein synthesis, cpm × 10^{-6}	% C	DNA synthesis, cpm × 10^{-3}	% C
Control	7.2 ± 0.6	100	22.46	100	3.85 ± 0.4	100
PDGF	18.3 ± 1.9	254	27.52	123	3.90 ± 0.8	101
PF 4	8.5 ± 0.7	118	22.54	100	ND*	—

Fibroblasts were cultured in the absence or presence of 100–250 ng of PDGF per ml (nine cultures) or 100–250 ng of PF 4 per ml (nine cultures) for comparison of collagenase-stimulatory activities, total protein synthesis, and DNA synthesis. The data are expressed as means ± SEM and as a percentage of the amount in control cultures containing no factor (% C).
*ND, assay not done.

Table 3. Effect of PDGF on translatable mRNA in human skin fibroblasts

Culture conditions	Culture medium: Collagenase activity, cpm/ml	Culture medium: Immunoreactive protein, ng/ml	Cell-free translation: Total protein synthesis, cpm × 10^{-3}	Cell-free translation: Immunoreactive collagenase, % of total
Control	9,520	1621	339.2	0.4
PDGF	20,640	3456	371.6	1.0

Fibroblasts were cultured for ≈22 hr in 1585-cm^2 roller bottles in the absence (control) or presence of PDGF (100 ng/ml), after which medium was harvested for collagenase activity and immunoreactive protein as a measure of stimulation in the intact cells. mRNA was then prepared for translation and determination of total protein synthesis by trichloroacetic acid precipitation and determination of collagenase synthesis by antibody precipitation.

clude, or be a prerequisite for, enhanced synthesis of collagenase.

The stimulatory action of PDGF places this molecule among a family of cytokines that modulate fibroblast function. Among these are interleukin 1 (39), a molecule of about 15 kDa that is produced by monocytes and by the epithelial cell line PAM 212 (40). This peptide stimulates collagenase production by rheumatoid synovial cells (41) and fibroblasts (42). A similar protein, a 20-kDa cytokine derived from epidermis and corneal epithelium, also stimulates collagenase synthesis in mesenchymal cells (43–45). Interestingly, it is likely that these agents also operate at a pretranslational level (46). Agents other than naturally occurring cytokines are also capable of inducing synthesis of collagenase *in vitro*. For example, several groups have shown that both phorbol esters (47–49) and urate crystals (48, 50) induce enhanced expression of collagenase in cultured rabbit synovial fibroblasts through a mechanism(s) that leads to enhanced translatable collagenase mRNA.

The demonstration that the natural cytokine, PDGF, has the capacity to stimulate the synthesis of a morphogenetically important enzyme such as collagenase may have broad biologic implications. Aside from the role of collagenase in would healing (15–18), this enzyme is important in vertebrate morphogenesis, development, and tissue differentiation (14). It is possible that PDGF may play a role in such events directly. Alternatively, PDGF might exert its effects by its close relationship to an oncogene product (51) or by an interaction with other oncogenes (52).

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