Differential Regulation of the Expression of Proteinases/Antiproteinases in Fibroblasts

EFFECTS OF INTERLEUKIN-1 AND PLATELET-DERIVED GROWTH FACTOR

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We have previously reported that polypeptide growth factors had an anti-inflammatory effect by decreasing the cytokine-enhanced expression of factor B (FB), an activator of the alternative complement pathway, in human fibroblasts. To further characterize the role of cytokines and growth factors in the inflammatory/repair continuum, we have studied the effects of interleukin-1 (IL-1) and platelet-derived growth factor (PDGF) on the expression of metalloproteinases/antiproteinases of the extracellular matrix in cultured human fibroblasts. Co-incubation of IL-1 and PDGF synergistically increased the expression of stromelysin and interstitial collagenase to 23-fold (for both proteins) over background, while PDGF decreased the IL-1-enhanced expression of FB by 82%. PDGF, but not IL-1, alone or in combination, increased the synthesis of tissue inhibitor of metalloproteinases. RNA blot analysis indicated that the changes in protein synthesis were regulated at a pretranslational level. Cycloheximide treatment indicated that the effects of PDGF on the metalloproteinases/antiproteinases were not protein-dependent, in contrast to results obtained for FB. The effect of the three dimeric forms of PDGF (AA, AB, and BB) on the synthesis of metalloproteinases and FB was also analyzed. The effects were qualitatively similar for each of the dimeric forms; however, the BB and AB isoforms had considerably greater effects than PDGF-AA. It has been reported that the PDGF receptors found in human fibroblasts have higher binding affinity for the BB and AB isoforms of the growth factor. The results presented in this paper are in accord with the possibility that differences in the biological activity of the three isoforms of PDGF are due to differences in the number or affinity of the binding sites of the target cells, rather than to different activation pathways of the receptor. Thus, PDGF increased cytokine effects on metalloproteinases, while decreasing cytokine effects on complement activator FB. The net effect of these changes may be to decrease inflammation and enhance remodeling early in repair and to enhance matrix stability later in the repair process.

Tissue response to an injury involves an orderly progression beginning with nonspecific inflammation, to specific degradation of pre-existing extracellular matrix, to repair (1). The nonspecific inflammation is marked by accumulation of neutrophils and mononuclear cells at the site of injury. Release of interleukin-1 (IL-1) and tumor necrosis factor (TNF) by mononuclear cells is thought to enhance this inflammatory response (2, 3). We have demonstrated that these cytokines stimulate synthesis of complement proteins, factor B (FB) and C3 by skin fibroblasts (4), providing a mechanism for enhancement of the antibody-independent alternative pathway of complement activation at the wound site.

Elucidation of the role of proinflammatory molecules, such as the alternative complement pathway, must involve not only knowledge of mechanisms that increase their synthesis at sites of inflammation, but also knowledge of mechanisms that decrease synthesis and thereby enhance (promote) resolution of the inflammation (5). Polypeptide growth factors, such as platelet-derived growth factor (PDGF), play a major role in repair of soft tissue damage, regulating cell migration, proliferation and differentiation, and accelerating wound healing and tissue regeneration (6–9). Recently, we provided evidence that polypeptide growth factors, PDGF, epidermal growth factor, and fibroblast growth factor, counter-regulate cytokine-enhanced synthesis of FB, and therefore have anti-inflammatory activity (10). Thus, growth factors, such as PDGF, which activate neutrophils and macrophages, and stimulate their directed migration in vitro (11, 12) and in vivo (6), have both proinflammatory and anti-inflammatory activities. These activities may be regulated temporally in injured tissues by other cytokines and growth factors.

The switch from inflammation to degradation of extracellular matrix, required in tissue repair to remove damaged tissue and permit neovessel formation, involves simulation of synthesis of a group of related metalloproteinases and their inhibitor. These enzymes, which include stromelysin and interstitial collagenase, and their inhibitor, tissue inhibitor of metalloproteinases, or TIMP, are synthesized in fibroblasts and macrophages (13–15). Like FB, synthesis of the metalloproteinases/antiproteinase is modulated by mediators of inflammation, such as cytokines and polypeptide growth factors (16–19).

The purpose of these studies was to delineate the influence...
on synthesis of fibroblast proteins by IL-1, a well recognized proinflammatory cytokine, and PDGF, a potent polypeptide growth factor that induces tissue repair processes. We report that the combination of PDGF and IL-1 increases synergistically the synthesis of stromelysin and interstitial collagenase. These synergistic responses are in contrast to the effects on synthesis of TIMP, which is increased only moderately by PDGF without further effect of IL-1, and the PDGF-induced inhibition of cytokine-enhanced expression of factor B. Thus, these results suggest a mechanism by which growth factors may promote a switch from acute inflammation toward processes of tissue remodeling and repair. Moreover, the study of the interaction of cytokines and growth factors in the differential regulation of metalloproteinases and complement proteins may provide a system for investigating mechanisms of signal transduction of both cytokines and growth factors.

MATERIALS AND METHODS

Cells—Human fibroblast lines were obtained from human Genetic Repository (National Institute of General Medical Sciences, Camden, NJ) (GM8399) or initiated from skin biopsies of normal volunteers. The cells were maintained in culture in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS) (GIBCO) at 37 °C in humidified air with 5% CO₂. Medium and FBS were both screened for low endotoxin content. Cells from passage 2 to 8 were used.

Biochemical Labeling—Human fibroblasts were grown to confluency in 24 well tissue culture plates (Corning) in DMEM containing 10% FBS. Before each experiment, the cells were washed twice with warm Hank's balanced salt solution (GIBCO) and incubated for 2 h in serum-free DMEM containing 0.1% bovine serum albumin (low endotoxin content, Sigma) in the presence of IL-1 and/or growth factor. The IL-1/1 (a gift from Dr. D. Perlmutter of Washington University, St. Louis, MO) and the BB, AA, and AB dimeric forms of PDGF (Angen Inc., Thousand Oaks, CA) were human recombinant DNA-derived, purified to homogeneity, and free of detectable endotoxin by the Limulus lysate assay. Each PDGF isoform preparation contained no detectable levels of the other two isoforms. Viability and number of cells, determined by trypan blue exclusion, were unaffected by any of the experimental conditions. Following incubation with cytokine and/or growth factors, the cells were washed twice with warm Hank's balanced salt solution and incubated for 1 h in DMEM without methionine (GIBCO) supplemented with 250 μCi/ml of [35S]methionine (ICN Radiochemical, Irvine, CA; specific activity, 1190 Ci/mmol) (20). At the end of the pulse period, the supernatant was recovered and the cells were washed with warm phosphate-buffered saline and lysed by one freeze-thaw cycle in the same buffer containing 0.5% sodium deoxycholate (Fisher), 1% SDS, 1% w/v Triton X-100, 2 mM phenylmethylsulfonyl fluoride (Sigma), 10 mM EDTA, and 100 μg/ml leupeptin (Behring Diagnostic Inc., Somerville, NJ). Lysed cells and media supernatant were clarified by centrifugation, and total protein synthesis was estimated by trichloroacetic acid precipitation of 5-μl aliquots of cell lysates or conditioned media.

Immunoprecipitation and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)—Polynonal antibody to complement protein FB was purchased from Atlantic Antibodies (Scarborough, ME), and antibodies to human collagenase, stromelysin, and TIMP were generated as described (15, 14, 21, 22). Sequential immunoprecipitation for FB, collagenase, stromelysin, and TIMP was performed as described (22, 23). Briefly, samples were incubated overnight at 4 °C with excess of the monospecific antibody. The immunocomplexes were precipitated with an excess of Staphylococcus aureus (strain A) (Immunoprecipitin, Bethesda Research Laboratories), washed, and subjected to SDS-PAGE analysis under reducing conditions (5% 2-mercaptoethanol) according to the method of Laemmli (24). Molecular weight standards were run in parallel lanes and visualized with Coomassie Brilliant Blue R-250 staining. The gels were fixed, impregnated with ENHANCE (Du Pont-New England Nuclear), dried, and exposed with an intensifying screen at −70 °C to Kodak X-Omat AR film (Eastman). To determine the incorporation of [35S]methionine into the individual immunoprecipitated proteins, gels slices corresponding to the autoradiographed bands, and gel slices for subtracting the background, were cut and digested in 15% hydrogen peroxide (18 h at 65 °C). Radioactivity was measured with Bio-Safe II scintillation fluid (RPI, Mount Prospect, IL) in an LKB liquid scintillation counter (model 1215).

RESULTS

Effects of PDGF-BB and IL-1 on the Expression of Metalloproteinases/TIMP and FB in Human Fibroblasts—To analyze the effects of IL-1 and PDGF-BB on the expression of stromelysin, collagenase, TIMP, and FB, confluent fibroblasts were incubated for 20 h in the presence of either IL-1 or PDGF-BB alone or the combination of IL-1 and PDGF-BB. The extent of synthesis of the specific proteins was determined by labeling the cells with [35S]methionine. The results are shown in Fig. 1, and the quantitative results of four separate experiments are presented in Table I. Three patterns of effects were apparent. For stromelysin and collagenase, IL-1 alone resulted in a moderate increase in expression, with less of an increase from PDGF-BB alone; the effect of the combination of the mediators was greater than the sum of the individual effects, indicating synergism between the mediators. Similar results were obtained when fibroblasts were incubated with TNFα, alone or in combination with the
The effects of different concentrations of PDGF-BB in association with a single concentration of IL-1 were also examined (Fig. 3). The results demonstrated a concentration-dependent increase in the synthesis of stromelysin and collagenase, with maximal synergy between IL-1 and PDGF-BB at the highest concentration of PDGF-BB used (Fig. 3, A and B). IL-1 did not alter the PDGF-BB effect on TIMP at any PDGF-BB concentration used (Fig. 3C). PDGF-BB counter-regulated the IL-1-enhanced synthesis of FB in a concentration-dependent manner (Fig. 3D).

Analysis of Steady State Levels of Specific mRNAs and Effect of Cycloheximide—Levels of specific mRNAs were quantitated by Northern blot analysis to determine whether cytokines and growth factors regulate the synthesis of metalloproteinases and FB at a translational or pretranslational level. Before lysis with guanidine, fibroblasts were incubated with IL-1 alone or with IL-1 in combination with PDGF-BB, with or without cycloheximide.

In general, mRNA results paralleled the changes in protein synthesis induced by IL-1 and PDGF-BB: for collagenase, both IL-1 and PDGF-BB increased mRNA levels, and the effect of the combination of mediators was greater than for either mediator alone (Fig. 4A); for TIMP, PDGF-BB, but not IL-1, increased mRNA, and the effect of the combination was similar to that of PDGF-BB alone (Fig. 4B); and for FB, IL-1 increased the mRNA level, and addition of PDGF-BB abrogated the increase (Fig. 4C). Results indicated that the cytokine and growth factor effects on synthesis of the proteins were all mediated at one or more pretranslational levels.

The effects of neither IL-1 nor PDGF on levels of collagenase and TIMP mRNA were altered by cycloheximide (Fig. 4, growth factor data not shown). For TIMP, PDGF-BB enhanced synthesis; IL-1 had no effect. Upon treatment with IL-1, a small decrease in the synthesis of this protein was observed in some experiments (Table I). The effect of the combination of mediators on the synthesis of TIMP was not greater than for PDGF-BB alone. For FB, as demonstrated previously (4, 10), IL-1 greatly increased the synthesis of FB. This IL-1 effect on FB was strongly inhibited by PDGF-BB, in contrast to the effects observed for stromelysin and collagenase.

The effects of PDGF-BB on the cytokine-induced changes in synthesis of the proteins occur throughout the IL-1 concentration-response range (Fig. 2). In the presence of IL-1 alone, synthesis of stromelysin and collagenase was increased maximally at a cytokine level of 0.1 ng/ml, while higher IL-1 concentrations were slightly less effective (Fig. 2, A and B). In contrast, the synthesis of FB increased almost linearly throughout the IL-1 concentration range (Fig. 2D). IL-1 had little or no effect on the expression of TIMP, at any concentration tested (Fig. 2C). As compared to the cytokine alone, higher doses of IL-1 in the presence of a constant concentration of PDGF-BB resulted in maximum synergism for enhancing stromelysin and collagenase synthesis (Fig. 2, A and B). The growth factor abrogated IL-1-enhanced FB production at all concentrations of IL-1 tested (Fig. 2D).

Confluent human fibroblasts were incubated for 24 h in the presence of different concentrations of IL-1 (closed circles) or IL-1 plus a single concentration of PDGF-BB (100 ng/ml) (open circles) or of PDGF-BB alone (closed triangle), then labeled for 1 h with [35S]methionine. Cell lysates were sequentially immunoprecipitated for the specific proteins and subjected to 12.5% SDS-PAGE (panels A, B, and C) or to 7.5% SDS-PAGE (panel D). Radioactive bands corresponding to the specific proteins were cut, digested, and counted for radioactivity. Results, calculated after subtraction of background counts/min, are expressed as experimental counts/min divided by control counts/min.

| Treatment | Stromelysin | Collagenase | TIMP | FB |
|-----------|-------------|-------------|------|----|
| IL-1      | 5.7 ± 0.3   | 7.3 ± 2     | 0.6 ± 0.06 | 37.4 ± 5 |
| IL-1 + PDGF | 23.0 ± 3.8  | 23.0 ± 5   | 3.1 ± 0.4 | 67.2 ± 2.2 |
| PDGF      | 2.3 ± 0.7   | 2.4 ± 0.1   | 2.9 ± 0.7 | 1.1 ± 0.04 |

* Data are expressed as -fold increase over the control, or experimental counts/min divided by control counts/min. Mean values of four separate experiments ± 1 S.D.

**IL-1 concentration = 0.5 ng/ml; PDGF concentration = 100 ng/ml.**
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Fig. 4. Effects of IL-1 and PDGF-BB on the levels of collagenase, TIMP, and FB mRNA. The cells were incubated for 20 h in the presence of medium alone as control (lane 1), IL-1 (0.1 ng/ml) (lane 2), IL-1 plus PDGF-BB (25 ng/ml) (lane 3), or PDGF-BB only (25 ng/ml) (lane 4). Equal amounts (10 μg) of total cellular RNA extracted with guanidium thiocyanate were electrophoresed on agarose gels and transferred to a nitrocellulose filter. The filters were hybridized with 32P-labeled cDNA probes, washed, and exposed to film. Lanes 1–4, cells incubated without cycloheximide; lanes 5–8, cells incubated with cycloheximide (5 μg/ml).

Fig. 5. Effects of IL-1 and of the AA, AB, and BB dimers of PDGF on the synthesis of collagenase and FB in human fibroblasts. Autoradiographs of 12.5% SDS-PAGE of collagenase (upper panel) and of 7.5% SDS-PAGE of FB (lower panel) immunoprecipitates from cells incubated for 20 h in the presence of medium alone (lane 1), IL-1, 0.5 ng/ml (lane 2), IL-1 plus PDGF-BB, -AA, or -AB, 10 ng/ml (lanes 3–5), or with PDGF-BB, -AA, or -AB alone, 10 ng/ml (lanes 6–8). Molecular mass markers are indicated.

A and B). For FB mRNA, the increased level induced by IL-1 was not affected by cycloheximide (Fig. 4C); however, the counter-regulatory effect of PDGF on the IL-1-enhanced level of FB mRNA was abrogated by cycloheximide (Fig. 4C). Thus, all of the effects of IL-1 and PDGF-BB on changes in levels of mRNA, only the counter-regulatory effect of PDGF-BB on FB appeared to require synthesis of new protein.

Effects of IL-1 and the Different Dimeric Forms of PDGF on the Synthesis of Metalloproteinases/TIMP and FB—The A and B chains of PDGF are secreted as AA, BB, and AB dimers. Receptors for both the A and B isoforms of PDGF have been identified on fibroblasts (30). In order to understand the physiological function of the three forms of PDGF, we compared the effects of the AB and AA dimers to the effects previously demonstrated for the BB dimer. The cells were incubated for 20 h in the presence of IL-1 or one of the PDGF dimers alone, or with a combination of IL-1 and one of the PDGF dimers (Fig. 5). The AB dimer had activity similar to the BB dimer, both qualitatively and quantitatively. Thus, AB increased synthesis of stromelysin and collagenase, acted synergistically with IL-1 for the synthesis of stromelysin and collagenase, and decreased the IL-1-enhanced synthesis of FB. The AA isoform had similar, but much reduced, activities. To further compare the relative efficiency of the three isoforms of PDGF, we performed concentration-response experiments in which fibroblasts were treated with concentrations of each isoform ranging from 1 to 100 ng/ml. Both the BB and AB dimers reached maximum activity at 10 ng/ml, while the AA dimer showed moderate activity only at 100 ng/ml (results not shown). The difference in the biological activity of the AA, AB, and BB forms of PDGF may be due to differences in the number of cell membrane binding sites, as it has been reported that human fibroblasts bind PDGF-BB and -AB well and PDGF-AA poorly (30).

DISCUSSION

Extraacellular matrix turnover and deposition are required in many biological processes, such as wound healing and steady state tissue remodeling. The regulation of extracellular matrix involves a balance between synthesis of its structural components and their degradation by metalloenzymes (stromelysin and collagenase), the activities of which are, in turn, modulated by the specific tissue inhibitor of metalloproteinases (TIMP) (31). Fibroblasts, as a site of synthesis of collagen and fibronecin, structural components of the extracellular matrix, as well as of the metalloproteinases/antiproteinases, play pivotal roles in repairing damaged connective tissues (32). They may also play roles in the earlier stages of the inflammatory process by synthesizing proinflammatory constituents, such as complement proteins (33).

In this report, we provide evidence that IL-1 and PDGF, normally released during the process of wound healing (5, 9), modulate the synthesis of metalloproteinases and complement proteins differently. While PDGF counter-regulates the cytokine-enhancing effect on the synthesis of FB, synthesis of metalloproteinases is synergistically increased by IL-1 and PDGF. As previously reported (19, 34), PDGF and IL-1 alone each induced small but significant increases in the expression of stromelysin and collagenase. The BB and AB, but not the AA, dimers of PDGF had quantitatively similar effects on expression of both the metalloproteinases and FB. These results are consistent with reports that type B receptor, which recognizes PDGF-BB and PDGF-AB, but not PDGF-AA, is more prevalent on fibroblasts than the type A receptor, which is believed to recognize all three dimers (30).

In contrast to alterations in the synthesis of collagenase and stromelysin, expression of TIMP was not significantly affected by IL-1, and only a small increase was observed after treatment with PDGF. Overall, these results suggest that IL-1 and PDGF preferentially increased the release of metalloenzymes relative to TIMP, providing fibroblasts with a matrix-degradative phenotype. Our results are consistent with those of MacNaul et al. (35) that IL-1 and TNF increased synthesis of stromelysin and collagenase (3–5-fold) much more than the synthesis of TIMP (1.2-fold) in human fibroblasts. This is in contrast with a previous report by Murphy et al. (36) using fetal lung fibroblasts, in which IL-1 produced parallel increases of TIMP and collagenase. It is possible that the differences in these results may be due to the cell line studied by Murphy et al. (36). The differences in the results of Murphy et al. (36) are not simply due to use of fetal cells, as preliminary studies in our laboratory indicate that regulation of collagenase and TIMP in fetal skin fibroblasts is similar to that observed in adult skin fibroblasts.

The molecular mechanisms responsible for regulation of
expression of metalloproteinase and complement protein genes have recently been studied. For example, both TNF and PDGF increase expression of the metalloproteinases by inducing synthesis of AP-1 (product of the jun protooncogene), which activates gene transcription directly by recognizing specific DNA sequences (37). The cellular pool of c-jun/AP-1 has been shown to be tightly correlated with the product of the c-fos oncogene (fos), suggesting that fos cooperates with AP-1 in activating the transcription of these genes, perhaps by enhancing the capacity of AP-1 to bind to DNA (37). Evidence has also been provided on the recognition and interaction of AP-1 with the 12-O-tetradecanoylphorbol-13-acetate-inducible enhancer elements in the 5' control regions of the human collagenase and homologous rat stromelysin genes (38, 39), suggesting that AP-1 plays a key role in mediating increases in the transcription of these genes induced by 12-O-tetradecanoylphorbol-13-acetate. Similarly, AP-1/fos DNA binding complexes play roles in the increased expression of stromelysin induced by PDGF (40).

We have observed that cycloheximide did not alter either the cytokine- or growth factor-induced changes in expression of the collagenase mRNA, suggesting that, under the conditions of our experiments, the effects of the IL-1 and PDGF on collagenase were not mediated by newly synthesized AP-1. However, residual protein synthesis of 5-10% that occurs in the presence of cycloheximide (4) may allow sufficient production of AP-1 and fos under conditions of overproduction of the mRNAs for these regulatory proteins. This mechanism has been hypothesized for the apparent discrepancies between the observations that regulation of collagenase by phorbol esters is not inhibitable by cycloheximide (39), and yet this induction requires new synthesis of fos (41). It is also possible that AP-1 is modified post-transcriptionally (41). The exact conditions of the experiment may also be important in determining the effect of cycloheximide, as Conca et al. (42) demonstrated that cycloheximide did inhibit IL-1-mediated induction of collagenase mRNA in fibroblasts that had been deprived of serum for 96 h; repletion of serum-induced collagenase mRNA and protein synthesis independent of IL-1. The findings of Brenner et al. (43) that cycloheximide blocks the stimulation of collagenase gene expression induced by TNF in cells not deprived of serum suggests that there are multiple mechanisms of collagenase regulation. Further studies need to be performed to clarify these issues.

Information has also been obtained on the molecular mechanisms which regulate the effect of cytokines on FB. The 5' regulatory region of factor B gene (BF) has been isolated and characterized (44). Recently, Nonaka et al. (45) have identified with the use of reporter genes and deletion constructs, the cis-acting DNA elements conferring the IL-1 and INF-γ responsiveness of BF. These sequences are located, at least in part, in the 3'-untranslated region of the C2 gene, about 400 bases pairs upstream of the BF RNA initiation site. The responsiveness to each mediator (IL-1 and INF-γ) is conferred by different upstream regions, which appear to have characteristic enhancer elements, since the position and the orientation of the enhancer sequences are irrelevant for the effect.

Our data and those currently available strongly suggest that regulation of proinflammatory constituents and modifiers of the extracellular matrix by growth factors, such as PDGF, at local sites may be important in enhancing tissue repair, by permitting earlier ingress of activated fibroblasts and endothelial cells into wounded tissue. Since PDGF accelerates healing of experimental dermal wounds (7, 8), PDGF appears to be an important promoter of matrix synthesis and stabilization. The balance between matrix degradation and matrix formation/stabilization induced by PDGF may be a function of its interaction with other cytokines, such as IL-1 within the injured tissue. IL-1 would be expected to be more prevalent early in tissue repair, during the inflammatory stage, when macrophages are attracted to the tissue in largest numbers. Other cytokines may influence the net effects of PDGF upon the matrix of healing tissues during the subsequent sequence of tissue repair.

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