Platelet-derived growth factors-AA and -BB regulate collagen and collagenase gene expression differentially in human fibroblasts

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Platelet-derived growth factor (PDGF) is a mitogen associated with tissue repair, a process involving collagen synthesis and remodelling by interstitial collagenase. This study examines and compares the regulation of interstitial collagenase and collagen gene expression by PDGF-AA and -BB in human fibroblasts. Time-course analysis showed that neither PDGF-AA or -BB had a consistent effect on the expression of pro- α 1(I) or pro- α 2(I) type-I collagen genes. In contrast, interstitial collagenase gene expression was found to be consistently up-regulated severalfold

by PDGF-BB. Enhanced expression of the collagenase gene was not apparently due to up-regulation of its promoter activity in human dermal fibroblasts, as indicated by transient and stable transfection experiments. Unlike PDGF-BB, PDGF-AA did not alter collagenase mRNA levels under low-serum culture conditions. Thus, the biological activities of the PDGF homodimers are different, with PDGF-BB being clearly more potent than PDGF-AA in its regulation of collagenase gene expression.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a serum mitogen that is implicated in wound healing and certain pathological conditions. The mitogen is composed of two homologous chains that are present as a dimeric structure [1,2]. The A- and B-peptides are encoded by two separate and independently regulated genes, but share ~ 60 % sequence similarity in their amino acid sequences. The three isoforms, PDGF-AA, -AB, and -BB, are synthesized as precursor molecules and undergo post-translational modifications [1,2].

PDGF is produced by various cells, including macrophages, endothelial and smooth-muscle cells. In the skin, both PDGF-AB and -BB isoforms are expressed by infiltrating mononuclear cells in the wound bed during injury, and by cells in the superficial dermis and epidermis [3]. The expression of PDGF and its receptors are reported to be highest during the initial phase of tissue repair [4].

PDGF has several biological functions. PDGF-AB or -BB are known to induce chemotaxis. *In vitro*, PDGF stimulates proliferation of fibroblasts, glial and vascular smooth-muscle cells [1,2]. *In vivo*, PDGF-BB enhances glycosaminoglycan deposition in incisional wound tissue [5]. *In vitro*, PDGF enhances synthesis of fibronectin, hyaluronic acid and collagenase [6–8].

Human dermal fibroblasts are known to express a variety of extracellular matrix macromolecules. In the present study, we report that human recombinant PDGF-AA and -BB do not regulate collagen gene expression, but alter collagenase gene expression differently in normal dermal fibroblasts and fibroblasts from keloids, a prototypic dermal fibrotic condition.

MATERIALS AND METHODS

Cell culture and growth factors

Human recombinant PDGF-AA and -BB (rPDGF-AA and -BB) were purchased from Austral Biologicals, San Ramon, CA,

U.S.A. Human dermal fibroblasts were grown from skin specimens obtained from reduction surgery. The donors did not present with any known systemic conditions. Fibroblasts were also established from keloid patients, whose ages matched those of the normal donors. Fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 200 units/ml of penicillin and 200 μ g/ml of streptomycin and glutamine.

Complementary DNA probes

The human cDNA probes that were used include a 1.5 kb pro- α 1(I) (HF-677) cDNA corresponding to the C-terminal pro-peptide and the C-terminal portion of the triple helical region of human pro- α 1(I) chain of type-I procollagen [9]; a 2.2 kb pro- α 2(I) procollagen cDNA (HF-1131) [10]; a 2.0 kb human collagenase cDNA [11]; a 1.3 kb glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [12]; and a cytoplasmic 7s cDNA [13].

Time-course and concentration-dependent studies

Early confluent cultures of normal and keloid fibroblasts were incubated with rPDGF-AA or -BB in DMEM containing 0.5 % FBS to minimize endogenous serum mitogens. For time-course studies, fibroblasts were incubated with 40 ng/ml of PDGF-AA or -BB for 24, 48, 72, 96 and 120 h. Concentration-dependent studies were performed with 0, 12.5, 25, or 50 ng/ml of PDGF-AA or -BB for 72 or 96 h.

Northern transfer analysis

Total RNAs were extracted from the cultures with 4 M guanidinium isothiocyanate, pH 7.0, containing 5 mM sodium citrate, 0.5 % (w/v) sarkosyl and 0.1 M 2-mercaptoethanol, and purified by CsCl density-gradient ultracentrifugation. The RNAs were electrophoresed in 1 % agarose gels under denaturing conditions and processed for Northern blotting [14].

Abbreviations used: PDGF, platelet-derived growth factor; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; TGF- β 1, transforming growth factor- β 1.

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Hybridizations were performed with human cDNA probes that had been radiolabelled by nick translation with [32 P]dCTP [15]. Prehybridization and hybridization solutions for the zeta-probe membranes consisted of 0.25 M NaCl, 0.25 M sodium phosphate, 50% formamide, 7% SDS and 1 mM EDTA, pH 8.0. Hybridizations were conducted at 42 °C for 24 h. The filters were washed several times at 65 °C, with a final stringency wash of $0.1 \times$ sodium saline citrate containing 0.1% SDS. The membrane filters were exposed to X-OMAT-AR films between intensifying screens (Kodak, Rochester, NY, U.S.A.), and the autoradiograms were scanned with a helium-neon laser densitometer (LKB, Bromma, Sweden). Collagen mRNA and collagenase mRNA levels were normalized with GAPDH or 7s RNA levels.

Stable and transient transfection/chloramphenicol acetyltransferase (CAT) analyses

Early confluent dermal fibroblast cultures were transfected with 30 μ g of plasmid DNA (pCLCAT3), which contains ~ 3.8 kb of the 5'-flanking DNA of the human collagenase gene linked to a CAT reporter gene [16]. The negative control was a pBSOCAT construct that lacks the promoter. Transient transfections were conducted using either the calcium phosphate/DNA precipitation method [17] or with a lipofectin reagent (GIBCO-BRL, Gaithersburg, MD, U.S.A.). Stable transfectants expressing the human interstitial collagenase promoter were prepared by transfecting NIH 3T3 fibroblasts with 20 μ g of pCLCAT3 and 2 μ g of a pRc/CMV vector containing a neomycin resistance gene (Invitrogen, San Diego, CA, U.S.A.). The stably transfected cells were then selected after growing the cultures in DMEM supplemented with 1 mg/ml of Geneticin (Boehringer-Mannheim). PDGF-BB was added to the cultures for 24, 48, or 72 h. Protein concentration of each sample was determined by a Bio-Rad dye-binding assay (Bio-Rad Lab, Richmond, CA, U.S.A.), and the same amount of extract, 10 μ g, from each sample was used for CAT assay. [14 C]Chloramphenicol was employed as a substrate [18]. The acetylated and non-acetylated forms of radioactive chloramphenicol were separated by TLC and visualized by autoradiography.

Influence of FBS on the regulatory effects of PDGF

PDGF is an endogenous mitogen present in serum. To determine the influence of FBS on the effects of PDGF-AA or -BB, early confluent fibroblasts were incubated with 0.5% or 5.0% FBS in the presence of 50 ng/ml PDGF-AA or -BB for 96 h. mRNA levels of collagenase and 7s from PDGF-treated cultures incubated in the presence of high and low FBS were determined by Northern analysis.

RESULTS

Time-course studies showed that the mRNA level of interstitial collagenase in normal dermal fibroblast cultures was marked during the initial 24 h in early confluent cultures, but was reduced over time (Figure 1). Keloid fibroblasts also expressed an abundant level of collagenase mRNA during early confluence, but the expression of collagenase gene was reduced as cellular density increased (Figure 2). The relative abundance of collagenase expressed by keloid fibroblasts was similar to that of normal donor fibroblasts.

Parallel cultures of normal or keloid fibroblasts were incubated with 50 ng/ml of PDGF-AA or -BB in medium containing 0.5% FBS for various periods. The interstitial collagenase mRNA

Figure 1 Time-course study of the effects of PDGF-BB on interstitial collagenase and type-I collagen gene expression by normal fibroblasts

Early confluent normal human dermal fibroblasts were incubated with 40 ng/ml of human recombinant PDGF-BB in DMEM and 0.5% FCS for 24, 48, 72, 96 or 120 h. Total RNA samples were isolated and prepared for Northern transfer analysis as detailed in the Materials and methods section. The typical transcript sizes for the pro- α 1(I) are 5.8 and 4.8 kb, pro- α 2(I) are 5.2 and 4.4, collagenase is 2.3 kb and 7s RNA is 0.2 kb. To ascertain the RNA loading, 7s cDNA was used to hybridize the filter. The filter was hybridized with a pro- α 2(I), collagenase and 7s probes first, then stripped and rehybridized with a pro- α 1(I) cDNA. The '+' denotes PDGF-BB treatment, while '-' represents parallel untreated controls.

Figure 2 Time-dependent regulation of collagenase and type-I collagen gene expression by PDGF-BB in keloid fibroblasts

Early confluent keloid fibroblasts were incubated without (—) and with (+) 40 ng/ml of PDGF-BB under identical culture conditions as in Figure 1 for the designated time periods. Northern analysis was performed with cDNAs specific for collagenase, pro- α 1(I) and pro- α 2(I) type-I collagen, as well as 7s RNA.

levels were increased dramatically by PDGF-BB in both normal and keloid fibroblast cultures (Figures 1 and 2). Up-regulation of collagenase gene expression by PDGF-BB was noted as early as 24 h (Figures 1 and 2). In normal dermal fibroblast cultures, PDGF-BB enhanced collagenase mRNA levels from 2- to 4.5-times above that of non-treated cultures over the course of 96 h. Similarly, in keloid fibroblast cultures, collagenase mRNA level was increased severalfold and maintained over a 96-h period. In contrast to PDGF-BB, PDGF-AA did not alter collagenase gene expression (data not shown).

Pro- α 1(I) collagen mRNA levels of keloid fibroblasts were increased greatly in comparison with that of age-matched normal fibroblasts. However, the relative amount of pro- α 2(I) collagen mRNA in normal and keloid fibroblast cultures was similar (Figures 1 and 2). Neither PDGF-AA nor -BB showed any consistent alteration of the pro- α 1(I) and pro- α 2(I) collagen

Figure 3 Concentration-dependent effects of PDGF-AA and PDGF-BB on the expression of collagenase and type-I collagen genes by normal dermal fibroblasts

Cultures in early confluence were incubated with 0, 12.5, 25, and 50 ng/ml of either PDGF-AA or PDGF-BB in DMEM and 0.5% FBS for 96 h. Total RNA was prepared for Northern transfer analysis and hybridized with collagenase, pro- $\alpha 2(I)$ type-I collagen and 7s RNA probes. The filter was also rehybridized with a pro- $\alpha 1(I)$ type-I collagen cDNA.

mRNA levels in normal or keloid fibroblast cultures (Figures 1 and 2).

In concentration-dependent studies, early confluent normal human dermal fibroblasts were incubated for 96 h with 0, 12.5, 25, or 50 ng/ml of PDGF-AA or -BB. PDGF-AA did not regulate collagenase or collagen gene expression (Figure 3) in the presence of 0.5% FBS. However, under the same culture conditions, PDGF-BB demonstrated a dose-response effect on the mRNA steady-state levels of collagenase. Collagenase mRNA level was enhanced severalfold with 25 and 50 ng/ml of PDGF-BB (Figure 3). Similarly, a concentration-dependent increase of 2- to 6.8-fold in collagenase mRNAs was noted in keloid fibroblasts incubated with PDGF-BB (data not shown).

Both pro- $\alpha 2(I)$ and pro- $\alpha 1(I)$ type-I collagen genes were not regulated by increasing concentrations of PDGF-AA or PDGF-BB (Figure 3). These findings support our previous time-course data which showed that type-I collagen gene expression was not altered by PDGF (Figure 3).

It is known that PDGF is a mitogen present in serum. Previous *in vitro* studies including our study have employed low con-

Figure 4 Influence of FBS on the regulatory effects of PDGF on the expression of collagenase

Normal fibroblasts were incubated in the presence of DMEM and 0.5% FBS (lanes 1–4) or 5.0% FBS (lanes 5–8) and 50 ng/ml of PDGF-AA (lanes 2 and 6) or PDGF-BB (lanes 4 and 8) for 96 h. Parallel control (C) flasks without PDGF treatment were included. Total RNA was isolated and hybridized with cDNAs specific for collagenase, pro- $\alpha 1(I)$ type-I collagen and 7s RNA.

Figure 5 Up-regulation of collagenase gene expression by PDGF in the presence of 5% FBS

Normal human dermal fibroblasts were incubated with 50 ng/ml of PDGF-AA or -BB in DMEM supplemented with 5% FBS for 24 and 48 h to determine the time of induction of collagenase gene expression by PDGF. The Northern filter was hybridized with collagenase and 7s RNA cDNA and overexposed to demonstrate the presence of the transcripts in the control (24 h) and the PDGF-AA (24 h treatment).

centrations of FBS [19]. To examine the potential synergistic effects of FBS and PDGF-AA or -BB on the expression of collagen and collagenase genes, early confluent fibroblasts were incubated with 50 ng/ml of PDGF-AA or -BB in the presence of 0.5% or 5.0% FBS for 96 h and Northern analysis was performed (Figure 4). PDGF-AA, in the presence of 5% FBS, elicited a perceptible increase in collagenase mRNA level compared with that at 0.5% serum, indicating a synergistic effect of serum (Figures 4 and 5). Stimulation of collagenase gene expression by PDGF-AA in the presence of 5% serum was noted as early as 24 h (Figure 5). PDGF-BB was effective, at low and high concentrations of FBS, in enhancing collagenase mRNA levels (Figure 4).

Transient transfections of fibroblasts with a collagenase promoter linked to a CAT reporter gene were performed to determine whether PDGF regulated promoter activity of the collagenase gene. At three time points of 24, 48, and 72 h of incubation with PDGF-BB, transfected fibroblasts showed no alteration in collagenase promoter activity (data not shown). In comparison, parallel fibroblast cultures incubated with transforming growth factor- $\beta 1$ (TGF- $\beta 1$) exhibited a major reduction in collagenase promoter activity, as reported previously [20].

NIH 3T3 fibroblasts were stably transfected with a human interstitial collagenase promoter and incubated with the PDGF isoforms. The results showed that the activity of the stably transfected collagenase promoter was not regulated by either PDGF-AA or -BB (data not shown).

DISCUSSION

PDGF is known for its mitogenic potential and is thought to be involved in wound healing and pathological conditions, such as atherosclerosis [21,22]. Increased turnover and remodelling of the extracellular matrix, involving collagens and interstitial collagenase, are common features of tissue repair. This study addresses the question of whether PDGF regulates matrix gene expression, specifically that of type-I collagen and interstitial collagenase genes. Regulation of type-I collagen and interstitial collagenase gene expression by PDGF-AA and -BB was compared in normal human dermal and keloid fibroblasts. The latter cell type was studied to determine whether keloid fibroblasts that over-express collagen are also regulated by PDGF.

Both PDGF-AA and -BB showed no consistent effect on the expression of pro- $\alpha 1(I)$ or pro- $\alpha 2(I)$ type-I collagen genes by

normal and keloid fibroblasts in concentration- and time-dependent experiments. A previous study also showed that PDGF had no effect on procollagen gene expression and synthesis in lung fibroblasts [19]. The results suggest that PDGF probably does not contribute to the direct deposition of collagen in normal tissue repair or in excessive healing that results in dermal fibrosis, as exemplified by keloids.

In the presence of low serum concentration, PDGF-AA had no discernible effect on the mRNA steady-state level of collagenase, but was capable of up-regulating collagenase gene expression when incubated with a higher serum concentration. The finding suggests the presence of synergistic activity of serum factor(s) with PDGF-AA to elicit increased collagenase gene expression. Unlike PDGF-AA, PDGF-BB was independently effective in up-regulating interstitial collagenase gene expression in the presence of low and high serum conditions. The results indicate that PDGF-BB is a more potent factor than PDGF-AA in the regulation of collagenase gene expression. PDGF-BB has also been reported to be a more potent mitogenic factor than PDGF-AA for vascular smooth-muscle cell growth [23,24], and more effective in promoting wound healing than PDGF-AA [25].

Our results using human recombinant PDGF support earlier studies which demonstrated that the addition of purified PDGF to vascular smooth-muscle cells and human dermal fibroblasts significantly enhanced the production of tissue collagenase/matrix metalloproteinase 1 (pro MMP-1) [8,26]. Our study documents that the regulation of collagenase expression by PDGF-BB is mediated at the pre-translational level. However, the detailed molecular mechanisms by which PDGF exerts its effect on collagenase gene expression are poorly understood. Our studies using transient and stable transfection experiments showed that PDGF-AA and -BB had no effect on collagenase promoter activity, suggesting that the elevated mRNA levels induced by PDGF-BB are not due to up-regulation of the promoter. It is possible that the increased collagenase mRNA levels in PDGF-treated fibroblast cultures may be explained by post-transcriptional/pre-translational modifications by PDGF. Parallel transfected cultures incubated with TGF- β 1 showed that the collagenase promoter contained responsive elements for TGF- β 1, as shown by notable suppression of the collagenase promoter activity after 24 and 48 h of PDGF treatment. However, it would appear that there were no apparent responsive elements for PDGF.

PDGF stimulation is known to signal early events, which include phosphatidylinositol breakdown and phosphorylation. PDGF has been documented to stimulate c-fos and c-myc transcription [27]. Fos proteins play a key role in signal transduction and are implicated as *trans*-activating and repressing molecules, regulating genes, such as the collagenase gene, that contain AP-1 related DNA sequence [20,28]. It is, therefore, surprising that the collagenase promoter activity was not altered in our transiently and stably transfected fibroblasts that were incubated with human recombinant PDGF. Thus, the mechanism by which PDGF-BB up-regulates interstitial collagenase gene expression remains to be elucidated.

Both keloid and normal fibroblasts were responsive to PDGF-BB, as shown by major stimulation of the collagenase gene expression. It is apparent that the dermal fibrotic cells are

capable of up-regulating collagenase mRNA levels in the presence of PDGF.

In summary, our results suggest that PDGF does not appear to play a direct regulatory role in collagen expression by fibroblasts during wound repair or in fibrotic conditions when collagen synthesis is activated. Rather, PDGF-BB, given its potent stimulation of collagenase gene expression, may play a viable role in the remodelling phase of tissue repair, when interstitial collagenase activity is high, resulting in accelerated turnover of collagen.

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