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Restoration of down-regulated PDGF receptors by TGF-β in human embryonic fibroblasts

Enhanced response during cellular in vitro aging

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Abstract

The study of [^{125}I]PDGF-BB binding to normal human embryonic lung fibroblasts, quiescent when cultured at sparsity in the presence of minute concentrations of homologous PDS, reveals approximately 2×10^5 binding sites for PDGF per cell; this number significantly increases during prolonged quiescence of the culture. As late as 48 h after down-regulation of PDGF receptors, the cells restore only partially their capacity to bind PDGF, with aged cells (above CPD 45) responding more rapidly and efficiently than younger ones. TGF-β significantly enhances restoration of PDGF receptors and, in aged cells in particular, its presence results in total receptor recovery within 24 h, suggesting a concerted action of PDGF and TGF-β regulating the proliferation of human fibroblasts in tissue regeneration.

Key words: Platelet-derived growth factor; Platelet-derived growth factor receptor; Receptor down-regulation; Transforming growth factor-β; Cellular aging; Human embryonic fibroblast

1. Introduction

Human platelet-derived growth factor (PDGF), a potent mitogen for cells of mesenchymal origin, is a dimeric polypeptide occurring in three isoforms: the heterodimer AB and the homodimers AA and BB [1–3]. PDGF exerts its action by interacting specifically with two membrane receptors, termed α and β, inducing upon binding their homo- or heterodimerization. Thus, PDGF-AA binds only to $\alpha\alpha$ dimer, PDGF-AB to $\alpha\alpha$ or $\alpha\beta$, whereas PDGF-BB to all three receptor dimers ($\alpha\alpha$, $\alpha\beta$, $\beta\beta$) [2,4].

Extensive studies of PDGF binding to its receptors have shown that the number of PDGF receptors, the ratio of the two receptor types, a well as the dissociation constant (K_d) vary widely between different cell strains and lines [4,6–11].

In the present report we examine the binding profile of PDGF-BB, i.e. the isoform which binds to all classes of PDGF receptors, in a cell assay system especially developed for this purpose and based on a human embryonic lung fibroblast strain. In the presence of minute concentrations of homologous plasma-derived serum (0.05% PDS), these cells can be synchronized arrested in G_0/G_1 even at sparsity and are highly responsive to mitogens [13]. The resulting cell system provides a valuable tool in studying the action of human growth factors under conditions which approximate in vitro those prevailing in the wound [14]. Under these conditions we estimated the number of PDGF receptors present in the cells and found it to be significantly increased during prolonged quiescence. We also observed that the cells cannot fully restore their initial binding capacity for PDGF long after down-regulation of its receptors, whereas aged cells achieve a better receptor recovery in the same time periods.

We have previously found that transforming growth factor-beta (TGF-β), a well known multifunctional agent secreted from human platelets simultaneously with PDGF [5,12], stimulates DNA synthesis in sparse fibroblasts and also acts in synergism with PDGF, a phenomenon which persists throughout cellular in vitro aging [14]. In this report we show that this synergism at the proliferative level seems to reflect similar events at the binding level. In fact, TGF-β significantly enhances the recovery of PDGF receptors and this effect is more pronounced in aged cells. It appears that these two growth factors act in concert and that their respective interactions may decisively regulate cellular proliferation.

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Abbreviations: PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-beta; PDS, plasma-derived serum; CPD, cell population doubling; FBS, fetal bovine serum.

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2. Materials and methods

2.1. Growth factors

Human recombinant PDGF c-sis (PDGF-BB) ^{125}I-labelled with Bolton and Hunter reagent and human recombinant PDGF c-sis were obtained from Amersham (Buckinghamshire, UK); human recombinant PDGF-AA and TGF-β1 from human platelets from Sigma (St. Louis, MO); PDGF from human platelets from R&D Systems (Minneapolis, USA).

2.2. Cell culture

Human embryonic lung fibroblasts (Flow 2002) were cultured in MEM supplemented with 10% (v/v) FBS as previously described [13]. Cells were subcultured every 7 days at 1:2 split ratio and grown at 37°C in a humid atmosphere of 5% CO_2. To determine cell numbers, cells were harvested with a trypsin-citrate (0.25–0.3%) solution, suspended in MEM containing 10% (v/v) FBS and counted in an Improved Double Neubauer haemocytometer. Cell viability was determined after incubating the harvested cells with the vital stain Neutral red and the non-vital stain Trypan blue, respectively.

2.3. Synchronization of the culture and DNA synthesis assay

The cells were maintained quiescent arrested in G_0/G_1 by PDS deprivation as described [13]. Briefly, cells were plated in 35 mm dishes at 4×10^3 cells/cm^2 in MEM supplemented with 0.05% PDS, prepared from fresh human blood [13]. Four days later, with a medium change on day 2, the growth factor samples were added at the indicated concentrations along with 0.1 μCi/ml [*methyl*-^{3}H]thymidine (25 Ci/mmol, Amersham). 48 h later thymidine incorporation into newly synthesized DNA was measured.

2.4. Binding studies

Binding assays were conducted as previously described [14]. Briefly, cells were plated at $4–7 \times 10^3$ cells/cm^2 in 12-well plates in MEM containing 1% PDS. After 24 h the medium was changed to MEM containing 0.05% PDS and renewed every 48 h until the indicated day of the experiment. This subconfluent culture maintains a constant number of viable cells ($3–6 \times 10^3$ cells/cm^2, depending on the cell number initially plated) for more than two weeks. Binding of the indicated concentrations of [^{125}I]PDGF-BB incubated with the cells at 4°C for 3 h was estimated as described [14]. Non-specific binding values were determined for a narrow range of PDGF concentrations around K_d (typically, 0.5–1.5 ng/ml [^{125}I]PDGF-BB) in the presence of 50 ng/ml PDGF-BB. A greater excess of unlabelled PDGF-BB did not affect [^{125}I]PDGF-BB binding. Non-specific binding for the other [^{125}I]PDGF-BB concentrations used was calculated from the above values according to Bowen-Pope and Ross [8]. Scatchard analysis was performed using the Ligand programm by Munson and Rodbard [15] as modified by McPherson [16]. PDGF receptor numbers per cell were calculated from B_{max} values divided by the numbers of cells remaining viable after the binding assay procedure.

2.5 Down-regulation assay

Cells prepared as above for the binding assay, were incubated for 1 h at 37°C with the indicated PDGF-BB concentrations in binding buffer (MEM buffered with 25 mM HEPES at pH 7.4 containing 2.5 mg/ml BSA). Following extensive washing (PBS containing 0.01 mg/ml $CaCl_2 \cdot 2H_2O$, 0.01 mg/ml $MgSO_4 \cdot 7H_2O$ and 0.1 mg/ml BSA) we incubated the cells with or without TGF-β in binding buffer for the indicated time periods, allowing for restoration of their PDGF binding capacity. The recovery of PDGF receptors was estimated by the [^{125}I]PDGF-BB binding assay as above.

Note: During the long incubation period following TGF-β addition (up to 72 h) minor cell number differences are observed, which do not interfere with the estimation of PDGF binding reported here.

3. Results and discussion

3.1. Characteristics of PDGF binding to human embryonic fibroblasts

As shown in Fig. 1A, specific binding of [^{125}I]PDGF-BB to young (CPD 21) human embryonic lung fibroblasts reached saturation at a ligand concentration of approx. 5 ng/ml. A linear Scatchard plot was obtained, indicating the presence of a single class of receptors with an apparent K_d of 50 pM (Fig. 1B). Nevertheless, as shown in Fig. 2, the AA dimer had a very low, yet distinct stimulatory effect on DNA synthesis, suggesting the presence of a limited population of type α receptors besides that of the β type, a finding widely encountered in normal human fibroblasts [17,18] and also confirmed by us in cross-competition binding studies (not shown here).

Under the above conditions, i.e. synchronization at cell sparsity, the total number of PDGF receptors (both α and β) in early passage cultures (CPD 21) was estimated to be approx. 2×10^5 per cell (Fig. 1B).

3.2. Prolonged quiescence increases PDGF binding in a manner similar to in vitro aging

Aged cells (CPD 49) bind significantly higher amounts of PDGF-BB in comparison to younger ones (CPD 21) due to a significant increase in receptor number (approx. 40%) as demonstrated by Scatchard analysis; no significant change in K_d was thereby observed (Fig. 1B).

Similarly, we noticed that higher amounts of PDGF-BB are bound following longer quiescence of the cells. Analysis of the binding data for cells of CPD 37 maintained quiescent for an additional week shows that increased binding is again a consequence of increased receptor levels (approx. 40%), with no significant change in K_d (Fig. 1C,D).

In similar experiments with human foreskin fibroblasts, Gerhard et al. recently reported higher PDGF receptor levels during in vitro aging which they ascribed to the increased surface area of aged cells [21]. An analogous explanation could also apply to the case of prolonged quiescence reported here, although no information is available to our knowledge on cell-size alterations during this state.

3.3. Time course of PDGF receptor recovery after down-regulation

Following binding, PDGF receptors dimerize, cluster in coated pits and become internalized, resulting in their down-regulation, a process which has been already well characterized [22–24]. In our system, after one hour of incubation at 37°C with excess PDGF-BB, we found a 80–85% reduction of [^{125}I]PDGF-BB binding capacity (Fig. 3).

As indicated by the kinetics of receptor recovery, young cells gradually increase their PDGF receptor levels, beginning 18 hours after down-regulation, but finally regain less than 50% of their initial binding capacity (48 h, Fig. 3A). In contrast, as shown in Fig. 3B, restoration of cell-surface PDGF receptors in aged cells begins approximately at 12 h and is significantly higher.

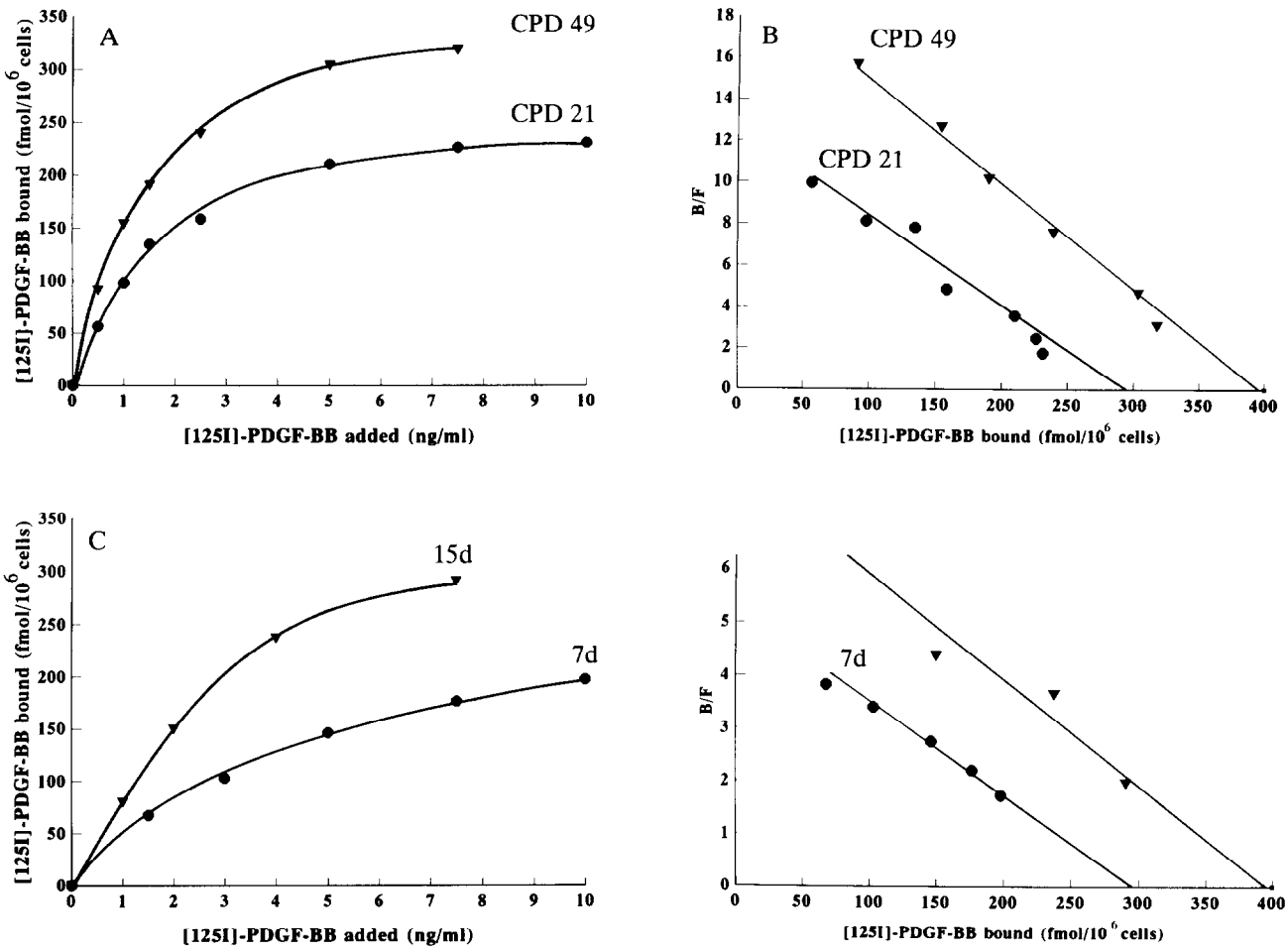

Fig. 1. (A) Effect of in vitro aging on PDGF binding. Cells of CPD 21 and 49 were prepared for binding assay as described in section 2. On day 5 after plating the cells were incubated with the indicated concentrations of [^{125}I]PDGF-BB. Bound PDGF-BB was measured as described. (B) Scatchard plots of binding data in A. The number of viable cells of CPD 21 and CPD 49 after the binding assay procedure was 2.4×10^4/well and 1×10^4/well, respectively. (C) Effect of prolonged quiescence on PDGF binding. Cells of CPD 37 were incubated with the indicated concentrations of [^{125}I]PDGF-BB on day 7 (7d) and 15 (15d), respectively, after initial plating. PDGF binding was estimated as above. (D) Scatchard plots of binding data in C. Number of viable cells: 4.3×10^4/well and 3.6×10^4/well for 7 day (7d)- and 15 day (15d)-old culture, respectively.

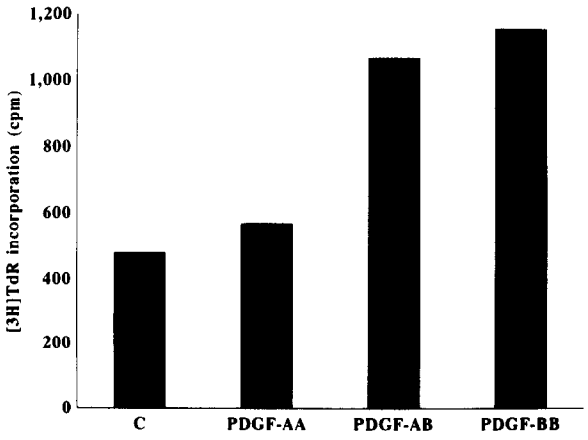

Fig. 2. Stimulation of DNA synthesis by PDGF isoforms. Cells of CPD 32 were synchronized as described in section 2. On day 4 after plating, 0.05% PDS in MEM was added containing: PDGF-AA (10 ng/ml); PDGF-AB (10 ng/ml); PDGF-BB (10 ng/ml). After 48 h [^{3}H]thymidine incorporation was measured. 0.05% PDS in MEM was used as control (C).

Normally, fibroblasts are exposed in vivo to extremely low concentrations of PDGF present in the human interstitial fluid [25]. In tissue lesion, platelets aggregate and subsequently release their contents resulting in high local concentrations of PDGF which probably could induce down-regulation of its receptors. However, as our in vitro results suggest, the cells need extremely long time periods (over 48 h, Fig. 3) to regain their capacity for full response to PDGF. This slow process may serve as a negative regulatory mechanism preventing an uncontrolled proliferation induced by the excessive supply of PDGF. Conversely, the ability of older cells to restore their PDGF receptors more rapidly (Fig. 3B) may partially counterbalance their markedly diminished proliferative response to PDGF [13,26].

Similar levels of restoration were reported recently in human foreskin fibroblasts of intermediate passages by Eriksson et al. [27]. In these cells the recovery begins as early as 8 h after addition of 20 ng/ml PDGF-BB and

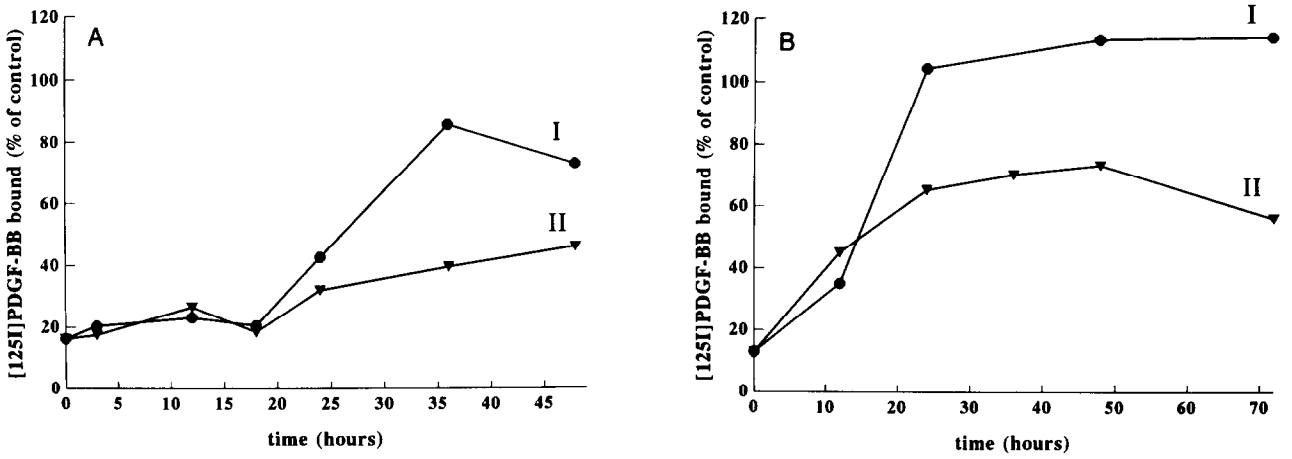

Fig. 3. Effect of TGF-β on the restoration of PDGF receptors. (A) Cells of CPD 34 were plated at 3.1×10^4 cells/well. After 5 days, they were incubated with 20 ng/ml PDGF-BB for 1 h at 37°C, followed by an incubation for the indicated time periods (I) with or (II) without 2 ng/ml TGF-β. Subsequently, the binding of 1 ng/ml [^{125}I]PDGF-BB was measured as in Fig. 1. 100% refers to 610 cpm of bound [^{125}I]PDGF-BB. Non-specific binding was not subtracted. (B) Cells of CPD 48 were plated at 3.1×10^4/well and further treated as in A. 100% refers to 902 cpm of bound [^{125}I]PDGF-BB.

reaches a plateau after 12 h, an effect which the authors ascribe to enhanced PDGF-receptor biosynthesis stimulated by the BB dimer [27]. This more rapid recovery could be explained by the fact that those measurements were performed in the continuous presence of PDGF, whereas in the experiments we report here the latter was completely removed from the culture milieu right after down-regulation, in order to obtain a rate of recovery unaffected by external growth stimuli.

3.4. TGF-β stimulates the restoration of PDGF-receptors

When incubated in the presence of TGF-β, young human embryonic fibroblasts with down-regulated PDGF receptors, showed significantly higher rates of recovery than untreated cells, reaching approximately 80% of their initial binding capacity for PDGF within 48 h (Fig. 3A). Intriguingly, this stimulatory effect of TGF- β was more pronounced in aged cells: 24 h after down- regulation, TGF-β-treated cells of CPD 48 fully restore their initial binding capacity for PDGF-BB (Fig. 3B). Hence, it appears that PDGF binding as well as the efficiency of the PDGF-receptor restoration mechanism are increased during cellular aging. These findings further support the suggestion that diminished proliferative response observed of aged cells to PDGF [13,26] is not due to scarcity of receptors but rather to events occurring downstream in the signal transduction cascade to the nucleus [21,28].

TGF-β has already been reported to modulate positively or negatively the expression of PDGF receptors in 3T3 mouse fibroblasts [19], as well as in human skin [11,20] and foreskin [11,29,30] fibroblasts. Our results suggest that after depletion of cell-surface PDGF binding sites, TGF-β actually restores the ability of human fibroblasts to bind and respond to PDGF, thus potentiating the proliferative action of the latter. This is further supported by our recent finding that the two growth factors act synergistically in stimulating DNA synthesis of cells cultured at sparsity under conditions roughly simulating in vitro those prevailing in the wound [14]. Since PDGF and TGF-β are contained in, and simultaneously released by platelets upon tissue lesion, it seems likely that the two factors may act in tandem rather than in a segregated manner, thus controlling by their concerted activities the proliferation of human fibroblasts.

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