Biochimica et Biophysica Acta, 971 (1988) 351–357
Elsevier

BBA 12319

Effect of the growth conditions on the expression of cell-surface-associated platelet-derived growth factor receptors in mouse fibroblasts

Renata Zippel [a], Laura Morello [a], Luisella Toschi [a], Lilia Alberghina [a], Paolo M. Comoglio [b] and Emmapaola Sturani [a]

[a] *Dipartimento di Fisiologia e Biochimica Generali, Università di Milano, Milano and* [b] *Dipartimento di Scienze Biomediche ed Oncologia Umana, Università, Torino (Italy)*

(Received 3 May 1988)

Key words: Platelet derived growth factor; Cell surface receptor; (Mouse fibroblasts)

The conditions affecting the appearance and disappearance of platelet-derived growth factor (PDGF) receptors from the pool of active cell surface-associated receptors were studied. Receptor molecules were revealed in intact Swiss 3T3 fibroblasts by their ability to bind ^{125}I-labeled PDGF and, due to their property to become phosphorylated in tyrosine following ligand binding, by antibodies to phosphotyrosine. PDGF receptor molecules were found to be quite scarce in exponentially growing fibroblasts as compared to quiescent cells. When growing cells were either shifted to a medium containing plasma or received suramin in the culture medium, cell surface-associated PDGF receptors largely increased. This process required about 12 h. Incubation of quiescent cells in serum, but not in plasma, induced a slow decrement of ligand-activatable receptors. In the presence of PDGF the rate of receptor removal from the cell surface was very rapid and was a function of the PDGF concentration. Quiescent cells deprived of cell-surface receptors by incubation with PDGF reexpressed PDGF receptors in about 14 h.

Introduction

The receptor for the platelet-derived growth factor (PDGF) is a plasma membrane glycoprotein of 170 kDa found in fibroblasts, smooth muscle cells and in glial cells. Like other growth factor receptors, it is endowed with tyrosine kinase activity and itself becomes phosphorylated in tyrosine following ligand binding [1–7].

We have previously used antibodies against azobenzylphosphonate, that specifically crossreact with phosphotyrosine [8,9], to study the kinetics of tyrosine phosphorylation and dephosphorylation of the PDGF receptor in intact quiescent Swiss 3T3 fibroblasts [5] and of epidermal growth factor receptor in A_{431} cells [10]. The tyrosine phosphorylation of the PDGF receptor at 37°C is a transient event and the receptor is then rapidly dephosphorylated. Moreover, bound PDGF is internalized and degraded and a decrement of the PDGF binding capacity is observed upon incubation of cells in PDGF [11–13].

The tyrosine phosphorylated form of the PDGF receptor is present only in PDGF-triggered cells. Thus, we have detected cell-surface-associated PDGF receptor molecules, able to bind PDGF and to become phosphorylated in tyrosine, by

Abbreviations: PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's; medium; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Correspondence: E. Sturani, Dipartimento di Fisiologia e Biochimica Generali, Università di Milano, Via Celoria 26, 20133 Milano, Italy.

analyzing in Western blots with phosphotyrosine antibodies the extracts of cells treated for a short time with a saturating amount of PDGF [14–16]. In addition, ^{125}I-PDGF binding to intact cells under the different conditions has been determined.

The PDGF receptor has been generally studied in quiescent fibroblasts. However growing cells also have to interact with PDGF at each new cell cycle [17–19]. We therefore studied the expression of functional receptors during exponential growth. Moreover, we present information about the kinetics of appearance and disappearance of PDGF from the pool of cell-surface-associated activatable receptors as influenced by either supplying or withdrawing the growth factor.

Materials and Methods

Cells and growth conditions. Mouse Swiss 3T3 fibroblasts were routinely grown and tested for mycoplasma contamination as previously described [5]. In general, quiescent cultures were obtained after 7 days of growth in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Flow) with one medium change on the third day of growth. When specified, quiescent cells were also prepared by shifting sparse cultures to 1% serum for 24 h. In both cases, over 90% of the cell population was found to have a presynthetic DNA content, as indicated by DNA flow cytofluorometry. Exponentially growing cells were obtained by plating cells in either 10 or 20% serum. After 1 day the medium was substituted with fresh medium containing the same serum concentration, and the cultures were used 15–24 h later. Analysis of DNA distributions indicated that under these conditions the population was in a real exponential growth. Average duplication times were 17 h and 15 h, respectively, in 10% and 20% serum (see also Ref. 19).

In some experiments exponentially growing cultures, obtained as indicated above, either received 1 mM suramin in the same medium or were shifted to DMEM containing 5% platelet-poor plasma, whereas control cultures were maintained in 20% serum. 18 h later the control culture was still exponential, while the other two arrested in the G_1 phase.

Stimulation with either serum or platelet-poor plasma [5] or PDGF was performed in DMEM containing 0.1% bovine serum albumin (BSA).

Phosphorylation of PDGF receptors. The cultures in the different growth conditions (quiescent, exponentially growing, or subjected to the specified treatments) were stimulated for 8 min with a saturating concentration of partially purified PDGF (400 ng/ml), while parallel cultures were left untreated. Monolayers were then extracted, fractionated by SDS-PAGE, blotted on nitrocellulose and immunodecorated with phosphotyrosine antibodies [9] and ^{125}I-labeled protein A as previously described [5]. After autoradiography the intensity of the 170 kDa band was quantified by determining the ^{125}I radioactivity associated with it. The difference between the cpm of the 170 kDa band of PDGF-treated cultures and that of untreated cultures gave estimate of the amount of activatable PDGF receptors.

PDGF binding. Monolayers plated in 24-well trays and grown as reported in the different experiments, were transferred in ice and incubated for 5 min with 20 mM acetic acid/150 mM NaCl/0.25% BSA (pH 3.7) [20]. Cells were then repeatedly washed with binding medium [10] and incubated for 2 h at 4°C with gentle shaking in the presence of ^{125}I-PDGF (53000 cpm/ng, 6 ng/ml). Nonspecific binding (generally 15% of total) was determined by adding an excess of partially purified PDGF together with iodinated PDGF. At the end of the incubation, cell-associated radioactivity was determined according to Heldin [21].

PDGF. Highly purified PDGF was prepared from outdated human platelets (kindly supplied by AVIS, Milano) by chromatography on carboxymethylcellulose, Blue-Sepharose, Bio-Gel P 150 and HPLC [22]. The final PDGF preparation (ED_{50} for [^{3}H]thymidine incorporation, 3 ng) was iodinated by the chloramine T method [21]. PDGF purified through the Bio-Gel step (partially purified PDGF) was used in the phosphorylation experiments.

Phosphotyrosine antibodies. Antibodies against azobenzylphosphonate, that specifically crossreact with phosphotyrosine, were prepared and purified as described [9].

Results

When quiescent Swiss 3T3 fibroblasts are treated for a few minutes with PDGF, the PDGF receptor becomes phosphorylated in tyrosine. In this form, the receptor was recognized as a sharp band of about 170 kDa in Western blots of total cellular extracts immunodecorated with antibodies against phosphotyrosine and ^{125}I-protein A [5], as shown in Fig. 1 lane 2. The ^{125}I radioactivity of protein A associated with the band was a function of the amount of PDGF receptors phosphorylated on tyrosine since, as shown in Fig. 2, it was proportional to the amount of stimulated cells analyzed. Thus, given a constant cell number, the band intensity depends on the average abundance of the phosphorylated receptor molecules in the population. Functionally active, cell surface-associated receptor molecules could, therefore, be estimated by their property to become phosphorylated in tyrosine following a short exposure to saturating PDGF concentration [14–16].

In Fig. 1, the presence of activatable PDGF receptors was investigated in cells exponentially growing (lanes 3 and 4) and compared to that of quiescent cells (lanes 1 and 2). Two plates of Swiss 3T3 fibroblasts exponentially growing in 10% serum where shifted to DMEM containing 0.1% BSA; one was stimulated for 8 min with PDGF, while the second received no additions. The phosphorylated band of 170 kDa was present in exponentially growing cells treated with PDGF (lane 4); however, its intensity was much lower than that obtained analyzing a comparable number of quiescent cells (lane 2). No bands were detected in unstimulated cells either quiescent or growing.

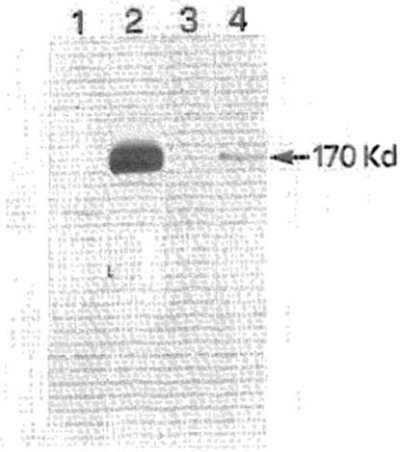

Fig. 1. Ligand-induced phosphorylation of the PDGF receptor during exponential growth and in quiescence. Quiescent (lanes 1 and 2) and exponentially growing fibroblasts (lanes 3 and 4) either received no additions (lanes 1 and 3) or were stimulated for 8 min with 400 ng/ml of partially purified PDGF (lanes 2 and 4). Aliquots of the extracts corresponding to $3 \cdot 10^5$ cells were fractionated by SDS-PAGE, blotted on nitrocellulose and immunodecorated with phosphotyrosine antibodies and ^{125}I-protein A. Autoradiography is shown.

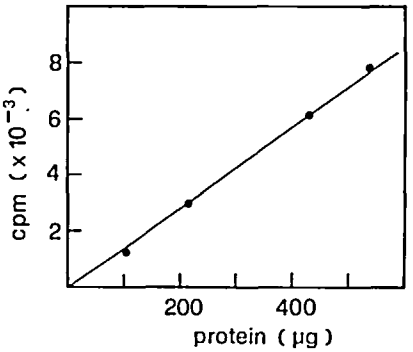

Fig. 2. The intensity of the 170 kDa band is proportional to the amount of cells analyzed. Quiescent Swiss 3T3 fibroblasts were stimulated with PDGF for 8 min. Monolayers were extracted and aliquots of the extract, corresponding to increasing amounts of protein, were analyzed as in Fig. 1. After autoradiography, the radioactivity of protein A associated with the 170 kDa band was counted.

In order to obtain an independent estimate of the amount of receptors, quiescent and exponentially growing fibroblasts were also tested for their ability to bind iodinated PDGF. For this experiment cells were grown in 24-well trays; to minimize problems connected with the adherance of PDGF to plastics, monolayers with the same cell density were used in both cases (about 20000 cells/cm^2). To obtain exponentially growing populations, cells were plated 48 h before the experiment and the medium was changed after 24 h. Quiescent cultures were obtained by starving for serum sparse cultures (1% serum for 24 h). Monolayers were then washed with a mild acidic solution, since it has been shown that this treatment rapidly removes PDGF bound to the cell surface [20,28] that might falsify the estimate based on ligand binding. Finally, the cultures were incubated at 4°C with ^{125}I-PDGF. While the bind-

TABLE I

BINDING OF ^{125}I-PDGF TO QUIESCENT AND EXPONENTIALLY GROWING CELLS

^{125}I-PDGF binding was determined on quiescent and exponentially growing cells as described in the text.

	Specific binding	
	fmol/10^6 cells	%
Quiescent	112±32	100
Exponential	22±11	20
Quiescent	80±11	100
Exponential	27± 2	34

ing capacity of quiescent cells was found to be 112 ± 32 and 80 ± 11 fmol of ^{125}I-PDGF/10^6 cells in two separate experiments, that of exponentially growing cells was 22 ± 11 and 27 ± 2 (Table I). Thus, two independent estimates indicate that during exponential growth, PDGF receptors are poorly expressed on the cell surface.

We reported previously [16,23] that when cells exponentially growing in the presence of 20% serum were deprived of PDGF by shifting them to a medium containing platelet-poor plasma the cell population completed a cycle and became arrested with a presynthetic DNA content in 18 h. The same results were obtained if PDGF binding to its receptor was prevented by the addition of suramin [14,24] in the serum-containing medium.

In addition to these findings, we now show that both treatments largely increase the PDGF binding capacity of the cells with respect to exponentially growing cultures maintained in the serum-containing medium. While the ^{125}I-PDGF bound by cultures exponentially growing in 20% serum was 24 ± 13 fmol/10^6 cells, the PDGF bound by cells shifted to plasma or treated with suramin was 120 ± 28 and 110 ± 20, respectively (Table II). Moreover, as shown in Fig. 3, a large increment of PDGF receptor molecules recognized by phosphotyrosine antibodies following PDGF stimulation, occurred during both treatments.

The time course of the reappearance of functional PDGF receptor molecules following the shift to plasma of exponentially growing cells is reported in Fig. 4. About 12 h were required for

TABLE II

EFFECT OF A TREATMENT WITH SURAMIN AND OF A SHIFT TO PLASMA ON THE ^{125}I-PDGF BINDING CAPACITY OF EXPONENTIALLY GROWING CELLS

Cultures exponentially growing in 20% serum were either maintained in serum or received 1 mM suramin or were shifted to a medium containing 5% plasma (as in Fig. 3). After 18 hrs the cultures were carefully washed, treated with acid (20) and ^{125}I-PDGF binding capacity was determined as described under Material and Methods.

	Specific binding	
	fmoles/10^6 cells	%
Exponential	24±13	100
Suramin	110±20	458
5% plasma	120±28	500

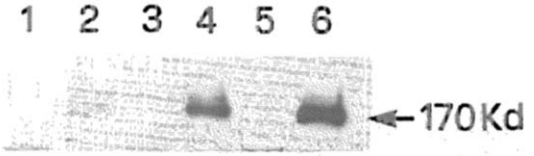

Fig. 3. Effect of a treatment with suramin and of a shift to plasma on the level of activatable PDGF receptors. Cultures exponentially growing in 20% serum were either maintained in serum (lanes 1 and 2), or received 1 mM suramin (lanes 3 and 4), or were shifted to a medium containing 5% platelet-poor plasma (lanes 5 and 6). 18 h later, the three sets of cultures were carefully washed (to remove suramin in particular) and either received no additions (lanes 1, 3, 5) or were stimulated with PDGF (lanes 2, 4, 6) and analyzed as in Fig. 1. Only the upper part of the gel is shown. In this experiment, the number of cells analyzed was $7.3 \cdot 10^5$ in lanes 1 and 2, $5 \cdot 10^5$ in lanes 3 and 4 and $5.2 \cdot 10^5$ in lanes 5 and 6.

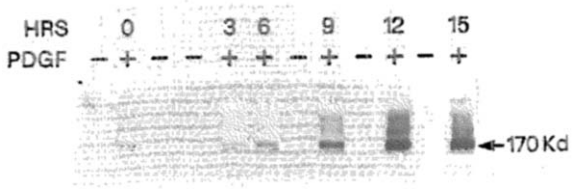

Fig. 4. Time course of the increment of activatable PDGF receptors following the shift of exponentially growing cells to plasma. Cells exponentially growing in 10% serum were washed and shifted to a medium containing 5% platelet-poor plasma (time zero). At time intervals, monolayers either received no further additions or were exposed for 8 min to PDGF and analyzed as already described. Only the upper part of the gel is shown.

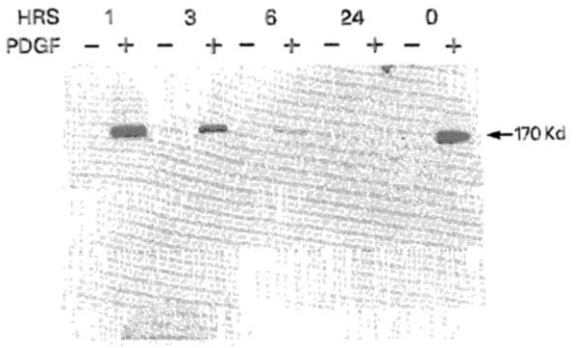

Fig. 5. Serum causes the decrement of activatable PDGF receptors in quiescent cells. Quiescent fibroblasts received 20% serum (time zero). At time intervals, monolayers either received no additions or were exposed for 8 min to PDGF and analyzed as described.

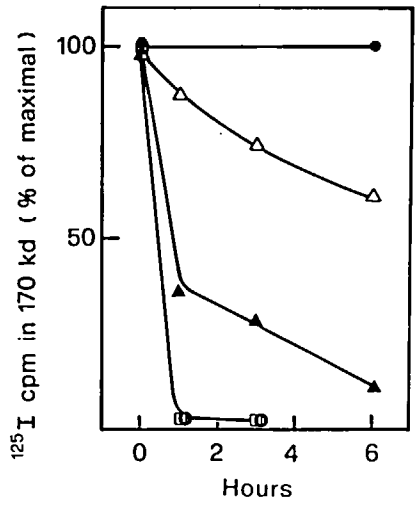

Fig. 6. Effect of plasma, serum and PDGF on the level of activatable PDGF receptors in quiescent cells. Quiescent fibroblasts were exposed either to 10% serum (△) or to 5% plasma (●) or to plasma together with 30, 100 or 300 ng/ml of partially purified PDGF (▲, □, ○, respectively). Before the onset of treatments (time zero) and 1, 3 and 6 h later, one set of cultures was stimulated with PDGF (400 ng/ml) for 8 min, while control cultures did not receive this latter addition. Cells were then analyzed as described. After autoradiography, the ^{125}I radioactivity of the area corresponding to the 170 kDa band was determined in both cultures that had received the 8 min stimulation with PDGF and in untreated control cultures. The differences between these two values were expressed as a percentage of that obtained at time zero and represent the relative amount of PDGF receptors that, at the different times of treatment, could be activated by PDGF addition.

the expression of functional receptor molecules at a level comparable to that found in quiescent cells.

These data suggest that some component of the serum present in the medium during exponential growth (most probably the PDGF) causes a continuous down-regulation of the receptors, keeping these molecules at a very low steady-state level.

This hypothesis is also supported by the observation that addition of serum to quiescent fibroblasts caused the slow, but complete, disappearance of PDGF receptors (Figs. 5 and 6): after 6 h in 20% serum most of the activatable PDGF receptors could no longer be detected by means of PDGF stimulation and analysis with phosphotyrosine antibodies. Addition of plasma to quiescent cells did not affect the level of receptors, while PDGF induced their rapid decrease in a dose-dependent manner (Fig. 6).

Quiescent cells in which PDGF receptors had been down-regulated by incubation with the ligand, again expressed the receptors on the cell surface if the cultures were shifted to plasma. The time required for full expression of the receptor was about 14 h after PDGF removal (see Fig. 7),

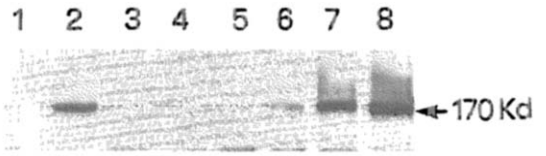

Fig. 7. Time course of the reexpression of activatable PDGF receptors in quiescent down-regulated cultures. Quiescent fibroblasts were exposed for 4 h to PDGF (300 ng/ml) to down-regulate PDGF receptors. Then monolayers were washed and transferred to the plasma-containing medium. At different times, one plate was stimulated for 8 min with PDGF for detection of activatable PDGF receptor, while another received no additions. Lane 1, quiescent cells, no additions; lane 2, quiescent cells stimulated for 8 min with PDGF; lanes 3 and 4, cells exposed for 4 h to PDGF (in lane 4, cells received a further 8 min stimulation with PDGF, while those in lane 3 received no further additions); lanes 5, 6, 7 and 8, cells were exposed for 4 h to PDGF, then shifted to plasma. At 4, 9, 14 and 19 h, respectively, after the shift, cells were stimulated for 8 min with PDGF. Controls of lanes 5, 6, 7 and 8, in which the 8 min stimulation with PDGF was omitted, were run on a different gel. In these samples the 170 kDa band was not visible (not shown).

very close to the time determined in exponentially growing cells deprived of PDGF. Addition of cycloheximide to the plasma-containing medium prevented the reappearance of receptors, (not shown) indicating that it depended on de novo protein synthesis.

Discussion

We have used two independent approaches to gain information on the expression of cell surface-associated PDGF receptors under different growth conditions. Immunoblot analysis with phosphotyrosine antibodies of PDGF-stimulated cells can give an estimate of functional receptor molecules, although, of course, it has to be pointed out that factors other than the number of PDGF receptors (such as differences in phosphatase activity or in stoichiometry of phosphate addition) may also influence the phosphorylation state of the receptor induced by PDGF treatment. In addition, we have determined the ligand-binding capacity of the cells. Since prebound PDGF might have masked receptor molecules already occupied, in PDGF binding studies cells were subjected to a mild acidic wash.

Very similar results were obtained from the two approaches so that some conclusion can be reached on the quantity of receptor present on the cell surface under the different growth conditions.

The interaction of cells with PDGF has been generally studied during quiescence; however, cells must interact with this growth factor even during continuous cycling, i.e., in exponential growth, otherwise they become growth-arrested [17–19,23].

As shown by the data reported above, the amount of functional receptor molecules able to bind PDGF and to become phosphorylated in tyrosine, was much lower in exponentially growing than in quiescent cells. Thus, the absence of PDGF receptors may be related to the rate of growth, in the sense that resting cells bear the PDGF receptors, while cycling cells do not. However the experiment reported in Fig. 7 shows that quiescent cells exposed to plasma after an initial PDGF treatment, reexpress the receptor on the cell surface. It is known that under these conditions cells leave G_1 and enter DNA synthesis [25–27]. So it seems more likely that the absence of PDGF receptor relates to the presence of PDGF in the medium rather than to growth rate.

The scarcity of receptor molecules observed in growing fibroblasts appears to be due to their continuous functional interaction with the PDGF present in serum, for the following reasons: (a) in quiescent cells, serum, but not platelet-poor plasma, caused a slow but complete down-regulation of PDGF receptors; down-regulation induced by PDGF was very rapid. (b) PDGF deprivation of growing cells, obtained by shifting the cultures to a medium containing plasma, enabled the appearance of receptors that could be phosphorylated in response to PDGF. (c) The same effect as that described for (b) was obtained by adding suramin to the growing cultures, which blocks binding of PDGF to its receptor and dissociates any PDGF which is already bound [24].

Thus, the low steady-state level of the cell surface-associated receptor observed during exponential growth appears to be the result of its rate of synthesis and of the continuous internalization caused by serum.

After PDGF removal, the time required for full expression of the receptors, both in exponentially growing cells and in down-regulated quiescent cells, was about 12–14 h, suggesting a similar rate of receptor synthesis under the two conditions.

The significance of PDGF receptor internalization to the production of mitogenic response is unknown. One may recall that relatively short exposure of quiescent fibroblasts to PDGF is sufficient to render them competent, i.e., able to respond to the set of factors present in plasma to enter the S phase [17]. Moreover, both acquisition of competence and receptor internalization depend on the PDGF concentration and on the time of exposure to it [25–27]. It will, therefore, be interesting to investigate whether the two events are correlated.

Acknowledgements

This work was supported by Italian CNR, Target Project Oncology Grant No. 87.001157.44115.08943 and by a Grant from AIRC (Italian Association for Cancer Research). L.M. is the recipient of a fellowship from AIRC. Suramin (Germanin-Bayer) was a kind gift of Bayer A.G.

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