Persistence of the Mitogenic Response to Platelet-derived Growth Factor (Competence) Does Not Reflect a Long-term Interaction between the Growth Factor and the Target Cell

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ABSTRACT Quiescent BALB/c-3T3 cells exposed briefly to platelet-derived growth factor (PDGF) become “competent” to replicate their DNA even if PDGF is removed from cell culture medium prior to the onset of DNA synthesis. We have suggested that persistence of the PDGF-induced competent state reflects a rapidly induced and relatively stable biochemical change within the target cells. Others suggest that the phenomenon reflects a long-term association between PDGF and its target cells or perhaps between PDGF and the cell culture dish. This controversy has been addressed (a) by examining the effect of anti-PDGF antibodies on PDGF-induced competence and (b) by studying the chemical fate of 125I-labeled PDGF.

Anti-PDGF antibodies inactive both soluble and surface-bound PDGF. However, if quiescent 3T3 cells are exposed to PDGF for as little as 30 min, subsequent addition of these antibodies to the culture medium does not prevent the mitogenic response. Under conditions where the PDGF-induced competent state decays stochastically with a $t_{1/2}$ of 18-20 h, cell-associated 125I-PDGF decays with a $t_{1/2}$ of ~50 min. These data do not support the concept that persistence of the PDGF-induced competent state reflects a long-term association between PDGF and the target cells or between PDGF and the culture dish.

Platelet-derived growth factor (PDGF) is a connective tissue mitogen that has been purified to homogeneity from clinically outdated human platelets (1-4). The salient feature of PDGF action is induction of a long-term cellular memory that we have termed “competence” (5). Quiescent BALB/c-3T3 cells exposed even briefly to PDGF become competent to replicate their DNA and divide. The PDGF-treated cell cultures do not “progress” efficiently through the G2/M phase of the cell cycle into S phase unless they are exposed continuously to a second set of growth factors contained in platelet-poor plasma (6); the “progression factors” in plasma appear similar to, and can be replaced by, epidermal growth factor (EGF) and insulin-like growth factors (somatomedins) (7, 8). In the absence of platelet-poor plasma, PDGF-treated cells remain competent to replicate their DNA but few do so. Readdition of plasma to PDGF-treated cells at later times allows expression of the mitogenic response.

The ability of PDGF to trigger a growth response long after it has been removed from the culture medium sets it apart from agents such as EGF, insulin, and the somatomedins which are required continuously in the culture medium to sustain a growth response (6-10). Somatic cell fusion analysis (11) together with other data (12) led us to suggest that persistence of the mitogenic response to PDGF in its physical absence from the culture medium reflected acquisition of a relatively stable cytoplasmic “second signal.” Other workers have challenged this view of PDGF action, opining that competence may simply reflect a long-term association between PDGF and its target cells (13, 14). Since purified preparations of PDGF adhere tenaciously to laboratory plasticware (15), it has also seemed possible that competence reflects the sustained action of PDGF adsorbed adventitiously to the cell culture dish.

In studies described here, we used anti-PDGF antibodies (16) to study the effects of transient exposure to PDGF under conditions which were not contingent upon the physical removal of PDGF from the culture dishes. We used 125I-PDGF.
to measure duration of the interaction between PDGF and BALB/c-3T3 cells under conditions where competence is induced. The data do not support the view that persistence of the PDGF-induced competent state reflects a long-term association between PDGF and the culture dish. By contrast, a large body of data is consistent with the view that competence reflects the intracellular accumulation of relatively stable PDGF-regulated gene products.

MATERIALS AND METHODS

Cell Culture: Stock cultures of BALB/c-3T3 cells (clone A-31) were maintained as previously described (5). All experiments were conducted on density-arrested monolayers of 3T3 cells which were prepared by seeding stock cell cultures in a density of ~10^4 cells/cm^2 and culturing them for 5 d in Dulbecco-Vogt modified Eagle’s medium supplemented with 10% calf serum.

PDGF: Human PDGF was purified to electrophoretic homogeneity by a modification of our previous protocol (1) that employs high performance liquid chromatography (HPLC) at two stages. Reverse phase chromatography on an HPLC C-18 column (Micromeritics Instruments Co., Norcross, GA) was used in lieu of the isoelectric focusing step described earlier. The PDGF was applied to the C-18 column in 0.1% trifluoroacetic acid, 2% acetonitrile, 6% isopropanol alcohol, and eluted with an isopropanol gradient to 36% final concentration. Molecular sieving chromatography on an HPLC sizing column (Waters Associates, Milford, MA) was employed in lieu of the SDS gel electrophoresis step described earlier (1). The solvent for the sizing chromatography step was 1 M ammonium acetate, 10% isopropanol alcohol. The revised procedure results in PDGF preparations that appear as a single band of 33,000 daltons by SDS gel electrophoresis and are mitogenic at protein concentrations of 0.5-1 ng/ml. One experiment was conducted with partially purified PDGF carried through the Biogel P150 step of our purification protocol (1). By multiple criteria, the mitogenic response of BALB/c-3T3 cells to pure and to partially purified PDGF is identical (1, 11, 12).

Radio-iodination: Human PDGF was labeled with ^125I by a modification of the Hunter-Greenwood procedure (17) as described by Singh et al. (18). Specific activity of the radio-labeled PDGF ranged from 0.2 to 0.3 mCi/mg PDGF in various preparations. The radio-labeling procedure did not alter the mitogenic activity of PDGF as assayed on BALB/c-3T3 cells under standard conditions (1).

Anti-PDGF: Rabbit anti-PDGF (16) was a generous gift from Dr. C. H. Heldin, Dr. A. Wassef, and Dr. B. Westerman of Upstate University. The antibody preparation was a Staph A-purified Ig fraction which at a protein concentration of 50 μg/ml would efficiently inactivate 10-12 ng/ml of pure PDGF. Control experiments demonstrated that (a) the anti-PDGF preparation itself had no mitogenic activity in our standard assay system (1), (b) that the anti-PDGF was not toxic to 3T3 cells, and (c) that nonimmune rabbit immunoglobulin had no anti-PDGF action.

RESULTS

Early Addition of Anti-PDGF Antibodies Is Required to Prevent the Induction of Competence

We used anti-PDGF antibodies to study the induction of competence under conditions that are not contingent upon physical removal of PDGF from the culture dishes. In the experiment summarized by Fig. 1, pure PDGF, at a final concentration of either 12 or 4 ng/ml, was added to quiescent density-arrested monolayers of 3T3 cells. At intervals thereafter, anti-PDGF was added directly to the tissue culture medium. As negative control, the PDGF was preincubated with antibody and the PDGF:anti-PDGF mixture was then added to the tissue culture cells. As a positive control, cells were incubated with PDGF alone. All of the cultures were incubated in the presence of [3H]thymidine and 5% platelet-poor plasma for 24 h, fixed, and processed for autoradiography. The data (Fig. 1) show (a) that the antibody preparation will neutralize ~12 ng/ml of PDGF within a 1-h period of time at 37°C, and (b) that if the cells are exposed to PDGF for even a few hours, the antibody cannot effectively block the subsequent mitogenic response.

In previous studies, it was shown that the length of time required for induction of competence was a function of PDGF concentration. At lower concentrations of PDGF, several hours exposure was required while at higher doses a 30-min "pulse" was effective. In these studies with antibodies, the PDGF at 12 ng/ml makes the cells "competent" for DNA synthesis more quickly than at 4 ng/ml. The antibody data are thus very similar to those described previously in which PDGF was removed by a medium change (5).

PDGF-induced Competent State Persists for Many Hours after Washout of PDGF

To accurately measure stability of the PDGF-induced competent state, we "pulsed" cells with PDGF and then transferred them to media containing a suboptimal (0.5%) concentration of plasma. At periodic intervals, the medium was supplemented with additional plasma to an optimal final concentration of 5%. The data (Fig. 2) show that few of the PDGF-treated cells enter S phase before the addition of optimal (5%) plasma. After the addition of 5% plasma to PDGF-treated cells, a lag time of 8 to 11 h precedes the onset of DNA synthesis. 24 h following the addition of plasma to PDGF-treated cells, the number of cells entering S phase has attained a maximum value. The percentage of competent cells is defined as this maximum value minus the "background" of cells that were not pulsed with PDGF and enter S phase in response to optimal (5%) plasma alone. With prolonged incubation in low plasma medium, some cells within the monolayer detach. A percentage of the remaining cells are stimulated by subsequent addition of optimal (5%) plasma in a phenomenon resembling the classic "wound healing response" (19). The "background" thus tends to increase with time, but with careful handling it can be held to <10%. We have found that for prolonged experiments 0.5% plasma facilitates cell attachment while not allowing optimum expression of the growth response to PDGF. For short-term experiments, 0.25% plasma as used previously is acceptable (5).

Data from the experiment outlined in Fig. 2 are replotted as the percentage of competent cells vs. incubation time in the
washed twice with PBS. The cells were subsequently incubated in
monolayer and TCA-soluble radioactivity in the cell culture
DME-0.25% plasma. At the indicated time intervals cultures were
assayed for autoradiography. The data are plotted as percentage of
unlabeled nuclei vs. time following the addition of plasma to 5%.
For simplicity's sake, only the data from the 0 delay, 8-h delay, and
16-h delay measurements are shown. Data from all six time points
are consolidated and replotted as shown in Fig. 3.

If 3T3 cells are incubated with PDGF for more than a few
hours, anti-PDGF antibodies cannot prevent induction of the
competent state; moreover, the PDGF-induced competent state
persists for many hours in the presence of anti-PDGF anti-
odies. These data could reflect the accumulation and long-
term retention of PDGF within a cellular compartment which
is inaccessible to antibodies. To determine the relationship
between stability of cell-associated PDGF and stability of the
PDGF-induced competent state, we exposed quiescent 3T3
cells to pure 125I-PDGF at 37°C under conditions paralleling
those employed for the experiments summarized in Figs. 2 and
3. At the end of 3-h incubation with 125I-PDGF at 37°C,
unbound radioactivity was removed by aspiration. The treated
monolayers were washed four times with PBS and the cultures
were subsequently incubated in DME-0.25% platelet-poor plasma.
At periodic intervals, the cell monolayers were har-

PDGF-induced Competence Persists in the
Presence of Anti-PDGF Antibodies

The salient feature of the PDGF-induced competent state is its
relative stability. The experiment summarized in Table 1 shows
that decay of the PDGF-induced competent state is unaffected
by the presence of anti-PDGF antibodies. The fraction of cells
which are rendered "competent" by a 3-h exposure to PDGF is
somewhat reduced when anti-PDGF antibodies are added after
the PDGF washout. However, subsequent decay of the competent
state in the antibody-treated cultures occurs at a comparable
rate. The differences calculated from these two point measure-
ments are probably not significant. The observation that fewer
cells are rendered competent when a 3-h "pulse" of PDGF is fol-
lowed by addition of anti-PDGF antibodies probably reflects the
fact that some PDGF remains associated with the cells for an hour
or two following wash-out from the tissue culture medium (see
below).

Cell-associated PDGF Is Degraded with a t1/2
of <1 h

Quiescent microtiter cultures of BALB/c-3T3 cells were exposed to
4 ng of pure PDGF in DME-0.5% platelet-poor plasma (40 µl) for 3 h at
37°C. PDGF-containing medium was then removed and the monolayers
were washed twice with phosphate-buffered saline. Half of the cultures
were transferred to 200 µl of Dulbecco's modified Eagle's medium supple-
mented with [3H]thymidine and an optimal (5%) concentration of platelet-poor
plasma with or without anti-PDGF antibodies (10 µl). The other half of the cultures
were transferred to medium containing a suboptimal (0.5%) concentration of
platelet-poor plasma with or without anti-PDGF antibodies. After 8-h incubation
in suboptimal plasma, the latter cultures received a supplement of platelet-
poor plasma to a final concentration of 5%. At 24 h after exposure to
5% plasma medium, all cultures were fixed with methanol and processed for
autoradiography. The data are shown as percent competent cells (as defined
in Fig. 2) after correction for the percentage of cells which became labeled
without exposure to PDGF (12%). The data points represent the mean of
experiments done in duplicate or triplicate.

### Table 1

| Plasma added at zero time | Plasma added after 8 h |
|--------------------------|------------------------|
| No AB                    | 87                     |
| +AB                      | 40                     |

Quiescent microtiter cultures of BALB/c-3T3 cells were exposed to 4 ng of pure
PDGF in DME-0.5% platelet-poor plasma (40 µl) for 3 h at 37°C. PDGF-
containing medium was then removed and the monolayers were washed
twice with phosphate-buffered saline. Half of the cultures were transferred
to 200 µl of Dulbecco's modified Eagle's medium supplemented with [3H]thy-
midine and an optimal (5%) concentration of platelet-poor plasma with or
without anti-PDGF antibodies (10 µl). The other half of the cultures were
transferred to medium containing a suboptimal (0.5%) concentration of
platelet-poor plasma with or without anti-PDGF antibodies. After 8-h incubation
in suboptimal plasma, the latter cultures received a supplement of platelet-
poor plasma to a final concentration of 5%. At 24 h after exposure to
5% plasma medium, all cultures were fixed with methanol and processed for
autoradiography. The data are shown as percent competent cells (as defined
in Fig. 2) after correction for the percentage of cells which became labeled
without exposure to PDGF (12%). The data points represent the mean of
experiments done in duplicate or triplicate.

### Figure 2

Decay of the PDGF-induced competent state. Quiescent density-arrested monolayers of
BALB/c-3T3 cells were exposed to 2 ng of 125I-PDGF in 200 µl of PDGF which had
been purified through the Biogel-P150 stage (1). This concentration of Biogel-
P150 PDGF corresponds to a protein concentration of
~250 ng/ml. Following 3-h exposure to PDGF, the culture
medium was aspirated and the monolayers were
washed twice with PBS. The PDGF-treated cell monolayers (●) and
also control cell cultures treated with solvent only (○) were subse-
sequently incubated with DME containing suboptimal (0.5%) platelet-
poor plasma plus [3H]thymidine (5 µCi/ml). At 0, 4, 8, 12, 16, and 20
h, platelet-poor plasma was added to PDGF-treated monolayers to
a final optimum concentration of 5%. At timed intervals following
the addition of platelet-poor plasma, cultures were fixed and proc-
essed for autoradiography. The data are plotted as percentage of
unlabeled nuclei vs. time following the addition of plasma to 5%.
For simplicity's sake, only the data from the 0 delay, 8-h delay, and
16-h delay measurements are shown. Data from all six time points
are consolidated and replotted as shown in Fig. 3.

### Figure 3

Stability of the PDGF-induced competent state exceeds
the stability of cell associated PDGF. Decay of the PDGF-induced
competent state (●) was determined from data of the experiment
described in Fig. 2. Percentage of competent cells is defined as the
fraction of PDGF-treated cells entering S phase without being exposed to PDGF. Stability of cell-associated PDGF (insert: note expanded time scale) was mea-
sured in the following way: Quiescent density-arrested monolayers of
BALB/c-3T3 cells were exposed to 2 ng of 125I-PDGF in 200 µl of
DME-0.25% plasma (equivalent to 50 U/ml used in Fig. 2). After 3
h, the culture medium was aspirated and the monolayers were
washed twice with PBS. The cells were subsequently incubated in
DME-0.25% plasma. At the indicated time intervals cultures were
harvested. The TCA-insoluble radioactivity associated with the cell
monolayer (●) and TCA-soluble radioactivity in the cell culture
media (○) were determined.
vested as well as the overlying cell culture medium. We monitored trichloroacetic acid (TCA)-insoluble radioactivity associated with the cell monolayer and TCA-soluble radioactivity associated with the culture medium. The data indicate that cell-associated PDGF is lost in exponential fashion with a \(t_{1/2}\) of ~50 min and that this loss is quantitatively accounted for by an increase of TCA-soluble radioactivity in the cell culture medium (Fig. 4, inset). By comparing the two decay curves shown in Fig. 4, it is clear that stability of PDGF-induced competence does not coincide with stability of cell-associated PDGF.

**Persistence of Competence Is Not Mediated by PDGF Adherent to the Surface or Side of the Culture Dish**

Purified PDGF adheres tenaciously to laboratory plasticware; moreover, surface-bound PDGF is mitogenically active (15). For these reasons, we wished to determine whether persistence of the PDGF-induced competent state was mediated by material adventitiously bound to the surface or sides of the culture dish. The experiments summarized in Fig. 4 and Table I argue against this possibility. The data show (a) that the amount of PDGF that binds to the surface of culture dishes containing 3T3 cell monolayers is quantitatively insufficient to account for competence and (b) that anti-PDGF antibodies (which block neither the induction nor the persistence of competence) inhibit surface-bound PDGF.

To determine the amount of PDGF which adheres to the surface of plastic culture dishes, we incubated \(^{125}\)I-PDGF at 37°C in empty cell-culture dishes and also in dishes that contained confluent monolayers of 3T3 cells. We measured the persistence of PDGF action because: (a) PDGF adherent to the side of culture dishes contributes an amount of mitogenic activity which is insufficient to account for competence, and (b) anti-PDGF antibodies block the mitogenic action of surface-bound PDGF.

FIGURE 4. PDGF adherent to culture dishes which contain 3T3 cell monolayers is quantitatively insufficient to account for competence. (\(\Delta\)) = PDGF as indicated to either unused empty microtiter culture wells or to identical wells containing BALB/c-3T3 cells which had been cultured to the confluent monolayer stage as previously described (5). The culture wells were incubated at 37°C for 1 h. Unbound PDGF was aspirated and the wells were washed five times with PBS. All wells were then resolubilized in 1 M NaOH. The data (Table II) show that anti-PDGF antibodies are capable of inactivating surface-bound PDGF.

**DISCUSSION**

Transient exposure to PDGF suffices to trigger the mitogenic response of BALB/c-3T3 cells in culture (5). Other workers have shown that transient exposure to PDGF induces growth of monkey arterial smooth muscle cells (20), human diploid fibroblasts (21, 22), and Swiss 3T3 cells (13). Thus, the phenomenon of the PDGF-induced "competent" state is broadly based although its biochemical basis has yet to be defined.

By inference from experiments conducted with TPA or with thrombin, Dicker and Rozengurt (13) and Van Obberghen-Schilling et al. (14) suggested that persistence of PDGF action following removal from the cell culture medium reflects a long-term association between PDGF and the target cell. The direct experiments reported here do not support such a conclusion. Anti-PDGF antibodies block the mitogenic action of surface bound (Table II) as well as soluble PDGF. Yet, when 3T3 cells are exposed even briefly to PDGF, subsequent addition of
PDGF treatment induces a prompt (within 40 min) accumulation of several cytoplasmic proteins (12), (d) PDGF regulates these proteins at a pre-translational level (27), and (e) variants of the 3T3 cell line which express these cytoplasmic proteins constitutively can proliferate in the absence of PDGF (12, 27).

PDGF is not the only regulatory agent which functions in a discontinuous manner on mammalian cells. Agents such as pituitary fibroblast growth factor (28), calcium phosphate (7), and the fibroblast-derived growth factor (13) mimic the action of PDGF rendering cultured fibroblasts competent to respond to a second set of growth factors and divide (7). A brief exposure to plant lectins renders mouse lymphocytes competent to replicate when secondary growth factors contained in serum are subsequently provided (29). In the erythropoietic pathway, transient exposure to soluble T cell factors primes undifferentiated erythroid precursor cells to respond to erythropoietin with proliferation and formation of “bursts” of erythrocytes (30–32). It would appear that PDGF is but one example of a class of regulatory signals which are needed only transiently to induce a mitogenic response. Thus insights into the molecular nature of “competence” in BALB/c-3T3 cells may take on broader significance in the area of cell growth regulation.

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