Platelet-derived Growth Factor

II. SPECIFIC BINDING TO CULTURED CELLS*

(Received for publication, September 4, 1981, and in revised form, November 25, 1981)

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We have prepared radioiodinated purified platelet-derived growth factor (125I-PDGF) which retains full mitogenic activity. The binding of 125I-PDGF to Swiss 3T3 cells is saturable and highly competed by whole blood serum, purified unlabeled PDGF, and by material from each stage in the purification of PDGF from platelet-rich plasma. Other purified mitogens and substances tested do not compete.

125I-PDGF binding to fibroblasts, 3T3 cells, and arterial smooth muscle cells shows an apparent dissociation constant of 10^-11 M, comparable to the range in which PDGF is mitogenic. A clone of Swiss 3T3 cells obtained from a population selected repeatedly against mitogenic response to PDGF shows a greatly reduced mitogenic response to PDGF and binds only 5% as much 125I-PDGF/cell. The binding capacity of the different cell types tested ranges from 2,500 binding sites/cell on the poorly responding variant to 390,000 binding sites/cell on one strain of Swiss 3T3 cells. Cell types that do not respond to PDGF do not show specific high affinity binding of 125I-PDGF. At 4°C, 125I-PDGF binding to monolayer cultures is relatively slow. Equilibrium binding of low concentrations of 125I-PDGF is not achieved during 7 h unless the binding medium is constantly mixed. 125I-PDGF binding at 4°C shows a broad pH optimum between 6.3 and 8.0. Binding does not seem to require Ca^2+ or Mg^2+ but is reduced more than 6-fold if both monovalent and divalent salts are omitted. The initial rate of 125I-PDGF binding is greater at 37°C than at 4°C but cell-associated 125I begins to decline soon after reaching a peak value at 30–60 min. Coincident with this decline, trichloracetic acid-soluble 125I appears in the medium and the binding capacity of the cells declines. These phenomena suggest that PDGF and its receptor may be internalized and degraded.

It has been known for many years that pure polypeptide hormones can profoundly alter the metabolic and mitotic activities of target cells. The mechanism(s) through which this alteration is accomplished remains obscure. Two different approaches have been taken in studying this problem. Some investigators have studied the final physiological effects of substances tested do not compete. 125I-PDGF binding to fibroblasts, 3T3 cells, and arterial smooth muscle cells shows an apparent dissociation constant of 10^-11 M, comparable to the range in which PDGF is mitogenic. A clone of Swiss 3T3 cells obtained from a population selected repeatedly against mitogenic response to PDGF shows a greatly reduced mitogenic response to PDGF and binds only 5% as much 125I-PDGF/cell. The binding capacity of the different cell types tested ranges from 2,500 binding sites/cell on the poorly responding variant to 390,000 binding sites/cell on one strain of Swiss 3T3 cells. Cell types that do not respond to PDGF do not show specific high affinity binding of 125I-PDGF. At 4°C, 125I-PDGF binding to monolayer cultures is relatively slow. Equilibrium binding of low concentrations of 125I-PDGF is not achieved during 7 h unless the binding medium is constantly mixed. 125I-PDGF binding at 4°C shows a broad pH optimum between 6.3 and 8.0. Binding does not seem to require Ca^2+ or Mg^2+ but is reduced more than 6-fold if both monovalent and divalent salts are omitted. The initial rate of 125I-PDGF binding is greater at 37°C than at 4°C but cell-associated 125I begins to decline soon after reaching a peak value at 30–60 min. Coincident with this decline, trichloracetic acid-soluble 125I appears in the medium and the binding capacity of the cells declines. These phenomena suggest that PDGF and its receptor may be internalized and degraded.

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A second tactic has been to investigate the initial interaction between hormone and cell with the hope that some characteristic(s) or direct result(s) of this interaction can be shown to be instrumental in effecting cell behavior. This interaction has been especially well studied for insulin (e.g. Cuatrecasas, 1971a) and epidermal growth factor (e.g. Cohen et al., 1975). These studies have demonstrated the existence of cell surface components (receptors) which bind hormone specifically and with high affinity. In this paper, we describe the binding of platelet-derived growth factor, one of the principal mitogens present in whole blood serum (Balk, 1971; Ross et al., 1974; Kohler and Lipton, 1974; Westermark and Wasteson, 1975), to cultured animal cells.

MATERIALS AND METHODS

RESULTS AND DISCUSSION

Effect of Iodination on the Biological Activity of PDGF—We have prepared an iodinated derivative of PDGF using the iodine monochloride method (see "Materials and Methods" for details). This iodinated PDGF contains an average of 0.25 molecule of 125I/molecule of PDGF (Table I, Experiment 3). The iodine monochloride method uses unlabeled 127ICl as an oxidizing agent (127ICl + 127I^{-} \rightarrow 127ICl + 127^{+} ) so that the specific activity of the 125I is necessarily reduced. As a result, each molecule of PDGF contains an average of 1.7 molecules of 125I + 127I and few molecules of PDGF would escape some extent of iodination.

The iodination of a peptide hormone could alter the binding or physiological properties of the hormone, as seems to be the case with insulin (Cuatrecasas, 1971a), epidermal growth factor (Cohen et al., 1975), platelet-derived growth factor (Ross et al., 1981), and others. This is the case with PDGF. We have prepared radioiodinated purified platelet-derived growth factor (125I-PDGF) which retains full mitogenic activity. The binding of 125I-PDGF to Swiss 3T3 cells is saturable and highly competed by whole blood serum, purified unlabeled PDGF, and by material from each stage in the purification of PDGF from platelet-rich plasma. Other purified mitogens and substances tested do not compete.

125I-PDGF binding to fibroblasts, 3T3 cells, and arterial smooth muscle cells shows an apparent dissociation constant of 10^-11 M, comparable to the range in which PDGF is mitogenic. A clone of Swiss 3T3 cells obtained from a population selected repeatedly against mitogenic response to PDGF shows a greatly reduced mitogenic response to PDGF and binds only 5% as much 125I-PDGF/cell. The binding capacity of the different cell types tested ranges from 2,500 binding sites/cell on the poorly responding variant to 390,000 binding sites/cell on one strain of Swiss 3T3 cells. Cell types that do not respond to PDGF do not show specific high affinity binding of 125I-PDGF. At 4°C, 125I-PDGF binding to monolayer cultures is relatively slow. Equilibrium binding of low concentrations of 125I-PDGF is not achieved during 7 h unless the binding medium is constantly mixed. 125I-PDGF binding at 4°C shows a broad pH optimum between 6.3 and 8.0. Binding does not seem to require Ca^2+ or Mg^2+ but is reduced more than 6-fold if both monovalent and divalent salts are omitted. The initial rate of 125I-PDGF binding is greater at 37°C than at 4°C but cell-associated 125I begins to decline soon after reaching a peak value at 30–60 min. Coincident with this decline, trichloracetic acid-soluble 125I appears in the medium and the binding capacity of the cells declines. These phenomena suggest that PDGF and its receptor may be internalized and degraded.

1 Portions of this paper (including "Materials and Methods," part of "Results," Figs. 4–8, and Tables II–IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 950 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-2177, cite the authors, and include a check or money order for $6.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations and trivial names used are: PDGF, platelet-derived growth factor; SIDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CMS-III, PDGF purified through the CM-Sephadex step of the procedure reported by Raines and Ross (1982); CMS-5, serum from which PDGF has been removed as described by Vogel et al. (1978); EGF, epidermal growth factor; FGF, fibroblast growth factor; EDTA, dose producing 50% of maximal effect; EdTA, (ethylenedinitrilotetraacetic acid; HEPES, 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; DVD, Dulbecco-Vogt modified Eagle's medium; F12, Ham's F-12 culture medium; TCA, trichloracetic acid.

* This work was supported by National Institutes of Health Grants HL18645 and AM13970, a grant to the Regional Primate Center (RR-00166), and a grant from R. J. Reynolds, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Effect of degree of iodination of PDGF on its binding characteristics and ability to stimulate \(^{3}H\)thymidine incorporation

For Experiment 1 six identical 1.0-μg samples of PDGF were iodinated as described under "Materials and Methods," except that the amount of total iodine/reaction mix was varied from 0.145 to 3.48 ng (column a). In each case, the ratio of total iodine/I\(^{125}\)I was maintained at 7.05. The values for molecules of I\(^{125}\)I incorporated/molecule of PDGF (column b) and for molecules of total iodine (I\(^{125}\)I + I\(^{127}\)I) incorporated/molecule of PDGF (column c) were calculated from the specific activity of the I\(^{125}\)I after equilibration with ICl. The binding characteristics of each preparation were determined using confluent cultures (3.8 × 10\(^5\) cells/2-cm\(^2\) well) of 3T3 cells preincubated in 2% calf CSM-I for 48 h. Binding was measured in duplicate at concentrations using 1 ml of binding medium/culture incubated for 4 h at 4°C with gentle shaking. The values of duplicate determinations were within 10% of their mean. Nonspecific binding (2.2% of total binding at concentrations of PDGF giving half-maximal binding) was subtracted. The concentration of PDGF needed for half-maximal specific binding (column d), and the maximum binding capacity of the cells (column e) were determined graphically from a plot of specific binding versus the concentration of PDGF remaining in the incubation medium at the end of the binding period. The ability of each preparation to stimulate \(^{3}H\)thymidine incorporation into parallel cultures was determined using parallel cultures incubated in duplicate with 8 concentrations of each PDGF preparation. After an 8-h incubation, \(^{3}H\)thymidine incorporation was measured and corrected for residual I\(^{125}\)I-PDGF binding as described under "Materials and Methods." The concentration of PDGF needed to produce half-maximal stimulation of \(^{3}H\)thymidine incorporation (column f) was determined graphically from a plot of \(^{3}H\)thymidine incorporation versus initial concentrations of PDGF. The maximum stimulation produced by each preparation was within 25% of the average maximum stimulation.

For Experiment 2, one 6.0-μg sample was iodinated as described under "Materials and Methods" for use in binding experiments, except that only 0.5 mCi of NaI\(^{125}\)I-PDGF was used. For Experiment 3 one 6.0-μg sample of PDGF was iodinated as described under "Materials and Methods" for use in binding experiments. For both experiments, a parallel 6.0-μg sample was processed, except that no I\(^{125}\)I or ICl was added. Measurements of binding and stimulation of \(^{3}H\)thymidine incorporation were as described for Experiment 1.

| Experiment | a. Total iodine in reaction mixture | b. Molecules I\(^{125}\)I incorporated/molecule PDGF | c. Molecules I\(^{125}\)I + I\(^{127}\)I incorporated/molecule PDGF | d. Concentration needed for half-saturation of binding | e. Maximum binding capacity | f. Concentration needed for half-maximal stimulation of \(^{3}H\)thymidine incorporation | g. Ratio, f/d |
|------------|------------------------------------|------------------------------------------|------------------------------------------|----------------------------------------|-------------------------------|----------------------------------------|----------------|
| 1          | 0.0145                            | 0.0069                                   | 0.48                                     | 21                                     | 430                           | 47                                                     | 2.2          |
| 2          | 0.29                              | 0.032                                    | 0.24                                     | 18                                     | 425                           | 29                                                     | 1.6          |
| 3          | 0.58                              | 0.062                                    | 0.44                                     | 16                                     | 310                           | 27                                                     | 1.7          |
| 4          | 1.16                              | 0.23                                     | 1.63                                     | 14                                     | 290                           | 28                                                     | 2.0          |
| 5          | 2.32                              | 0.32                                     | 2.26                                     | 25                                     | 230                           | 50                                                     | 2.0          |
| 6          | 3.48                              | 0.37                                     | 2.61                                     | 14                                     | 265                           | 28                                                     | 2.0          |
| 7          | 0.0                               | 0.0                                      | 0.0                                      | 25                                     | 250                           | 55                                                     | 2.2          |
| 8          | 6.35                              | 0.1                                      | 2.7                                      | 25                                     | 230                           | 50                                                     | 2.0          |
| 9          | 0.0                               | 0.0                                      | 0.0                                      | 14                                     | 265                           | 28                                                     | 2.0          |
| 10         | 0.96                              | 0.25                                     | 1.77                                     | 20                                     | 619                           | 42                                                     | 2.1          |

For each preparation of I\(^{125}\)I-PDGF, we also determined the percentage of radioactivity that is incapable of high affinity binding to Swiss 3T3 cells ("Materials and Methods," Fig. 4). The values for the percentage of total counts/min which cannot bind to Swiss 3T3 cells ranged from 40% for a PDGF preparation that contained a 38,000-dalton contaminant (visualized on silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and on autoradiograms of the iodinated preparation), to 18% for an I\(^{125}\)I-PDGF preparation showing small amounts of I\(^{125}\)I-gelatin and no other detectable contaminants. These values are comparable to values obtained after iodination of other purified hormones (Cuatrecasas and Hollenberg, 1979). The nonbinding material could represent material unrelated to PDGF, PDGF molecules altered during iodination, or incomplete quenching of the iodination reaction with consequent iodination of small amounts of the carrier protein (gelatin) used during dialysis. This type of information concerning the presence of radiolabeled nonbinding components is necessary for accurate Scatchard analysis. Scatchard analysis of binding data requires determination of the concentration of free ligand in solution at the end of the incubation period. Correction must be made for the presence of labeled nonbinding components, since their presence would contribute to the free counts/min without contributing to bound counts/min. This would raise the apparent dissociation constant. After correction for nonbinding radioactivity, all preparations of I\(^{125}\)I-PDGF used to date have given comparable values of apparent K\(_d\) for binding to Swiss 3T3 cells.

Determination of Cell-associated I\(^{125}\)I-PDGF—Before utilizing I\(^{125}\)I-PDGF to probe the interaction of PDGF with cultured animal cells, we had to demonstrate that, within our system: 1) the measured interaction is truly a cellularphenom-
Fig. 1. Comparison between [3H]thymidine incorporation and 125I-PDGF binding competition assays for PDGF. a, [3H]thymidine incorporation. Confluent cultures of 3T3 cells in Costar 24-well culture trays were incubated for 48 h in 1 ml of a 1:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 medium containing 2% calf CMS-I. Material from successive stages in the purification of PDGF was added and [3H]thymidine incorporation into trichloroacetic acid-insoluble material was determined 18 h later as described under "Materials and Methods." The results are plotted as the ratio of [3H]thymidine incorporation in test cultures/[3H]thymidine incorporation in cultures receiving addition of saline alone. The mean ± range of duplicate determinations is plotted. A, human whole blood serum; Δ, human platelet-rich plasma; ■, PDGF purified through CM-Sepharose; ○, PDGF purified through Sephacryl S-200 gel filtration; ●, PDGF purified through heparin-Sepharose chromatography; ○, PDGF purified through phenyl-Sepharose hydrophobic chromatography. Note that the preparations of PDGF used here were not derived from the stages in purification of PDGF from a single batch of platelet-rich plasma. Instead, they represent material available from several different preparations. b, competition for 125I-PDGF binding. Parallel cultures were used to determine competition for 125I-PDGF binding. Cultures were incubated for 4 h at 4°C with gentle shaking in binding medium containing 6 pM 125I-PDGF, plus the amount of test competitor indicated on the abscissa. Cell-bound 125I-PDGF was determined as described under "Materials and Methods." Binding has been plotted without correction for nonspecific binding (noncompetable binding) which can be seen from the curve for competition by CMS-III PDGF to be 1-2% of total binding. The symbols are the same as those in a. The mean ± range of duplicate determinations is plotted.

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125I-Insulin binding to talc shares many of the properties of 125I-insulin binding to cell surface receptors (Cuatrecasas and Hollenberg, 1975). In preliminary studies, we found that 125I-PDGF binds to bare culture wells and that this binding can be partially competed by unlabeled PDGF (see "Materials and Methods"). Since, in further studies, we planned to use competition by unlabeled PDGF as being diagnostic of receptor-bound 125I-PDGF, this was unacceptable. To circumvent this problem, we used a solution containing the nonionic detergent Triton X-100 to solubilize cell-associated 125I-PDGF without eluting dish-bound 125I-PDGF (for further details see "Materials and Methods"). A similar procedure was developed independently by Heldin et al. (1981).

Competition for 125I-PDGF Binding—As PDGF is increasingly purified from human platelet-rich plasma, smaller concentrations are sufficient to stimulate [3H]thymidine incorporation (Fig. 1a, Table II, and Raines and Ross, 1982). Fig. 1b and Table II show that the ability of low concentrations of partially purified preparations of PDGF to compete for 125I-PDGF binding is also increased as the mitogenic activity is purified. We have chosen to use PDGF purified through CM-Sepharose (CMS-III) to determine noncompetable binding in standard assays. At concentrations of 200-400 μg/ml, CMS-III reduces 125I-PDGF binding to 1-2% of binding in the absence of a competitor. Although competition to this extent was not achieved in Fig. 1b using the most purified preparation of PDGF, the slope of the competition curve suggests that higher concentrations of this preparation would have been as effective as high concentrations of CMS-III.

In order to determine whether competition for 125I-PDGF binding is specific for PDGF, we tested other mitogens, including EGF, fibroblast growth factor, and insulin, for their ability to compete for 125I-PDGF binding to 3T3 cells (Table II). Each of these mitogens was tested for ability to compete using concentrations at least 400-fold higher than the concentration required to produce half-maximal stimulation of [3H]thymidine incorporation. Several nonmitogens were also tested, including human thrombospondin and partially puri-

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FIG. 2. 125I-PDGF binding and stimulation of [3H]thymidine incorporation in different cell types. Each cell type was plated in Costar 24-well trays and grown to confluence in a 1:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 medium + 5% calf serum. The growth medium was then replaced with a 1:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 medium + 2% calf CMS-I for 48 h. A, 125I-PDGF binding was determined using triplicate cultures for each concentration by incubating with 1 ml of binding medium containing increasing amounts of 125I-PDGF. The cultures were incubated for 5% h at 4 °C with gentle agitation before measuring cell-bound 125I-PDGF as described under “Materials and Methods.” Nonspecific binding was determined as described under “Materials and Methods,” and has been subtracted (see Table III for values). Cell number was determined as described in the legend to Table III. The concentrations plotted on the abscissa represent the concentrations of intact 125I-PDGF at the end of the binding period, calculated as described under “Materials and Methods.” The cell types used were: ○, high binding Swiss 3T3; □, Swiss 3T3; △, monkey smooth muscle; ◊, human foreskin fibroblasts; ▲, human smooth muscle cells; ×, Swiss 3T3 variant clone PF 2; ■, A431 carcinoma. B, parallel cultures were incubated for 18 h with increasing concentrations of unlabeled PDGF before incorporation of [3H]thymidine into trichloroacetic acid-insoluble material was measured, as described under “Materials and Methods.” The results are plotted as fold stimulation (ratio of [3H]thymidine incorporation in the presence of added PDGF/ [3H]thymidine incorporation in the absence of added PDGF) versus the initial concentration of PDGF. [3H]Thymidine incorporation was not determined for human smooth muscle cells or A431 cells.

The mannose-binding lectin concanavalin A has been reported to reduce binding of 125I-EGF (Carpenter and Cohen, 1977) and 125I-insulin (Cuatrecasas and Tell, 1973) to their respective receptors. Concanavalin A did not significantly reduce 125I-PDGF binding in our system, suggesting that neither the receptor, nor PDGF, contains available α-mannose residues near regions involved in PDGF binding.

Gajdusek et al. (1980) have reported that serum-free medium conditioned by bovine aortic endothelial cells is mitogenic for 3T3 cells. The major mitogenic activity in endothelial cell-conditioned medium seems to be biochemically distinct from PDGF (Gajdusek et al., 1980). Endothelial cell-conditioned medium does show some ability to compete for 125I-PDGF binding to Swiss 3T3 cells but, until endothelial cell-conditioned medium is resolved into purified components, we will not know how similar this binding competitor activity is to PDGF. Heldin et al. (1980) have reported that a line of osteosarcoma cells produces a growth factor which may be identical to PDGF and that this factor is equipotent with PDGF as a binding competitor.

Cell Specificity of 125I-PDGF Binding—In order to demonstrate that 125I-PDGF binding is not just a general property displayed by cells in culture, as opposed to a specific property related to cell response to PDGF, we measured 125I-PDGF binding to cells which do not respond to PDGF in culture. As an example of a cell type without PDGF receptors, we have used the human cervical carcinoma cell line A431 (Fig. 2). This cell line displayed no measurable specific 125I-PDGF binding (Fig. 2A) and conditioned serum-free medium from this cell had no effect on [3H]thymidine incorporation into trichloroacetic acid-insoluble material and did not block 125I-PDGF binding (data not shown).

Low passage bovine aortic endothelial cells also showed no mitogenic response to addition of PDGF and showed no competable binding. However, since endothelial cells produce a competitor of 125I-PDGF binding, it is possible that these cells do have PDGF receptors but that these receptors are already occupied by an endogenously produced competitor. Such a situation does seem to exist for EGF receptors on Kirsten sarcoma-transformed cells (DeLarco and Todaro, 1980) and for PDGF receptors on one osteosarcoma cell line (Heldin et al., 1980, 1981). Because of this type of phenomenon, it is difficult to determine whether a cell type which produces a binding competitor has receptors for the test hormone.

D. F. Bowen-Pope and P. DiCorleto, unpublished observations.
A second type of evidence for the specificity of \(^{125}\)I-PDGF interaction with responsive cells has been obtained from affinity-labeling experiments. Using bifunctional cross-linking reagents, Glenn et al. (1982) demonstrated that \(^{125}\)I-PDGF bound to several PDGF-responsive cell types can be cross-linked to a single high molecular weight cell surface component. \(^{125}\)I-PDGF binding to this component displays the same properties as the specific \(^{125}\)I-PDGF binding measured by Triton X-100 extraction described in this paper. The authors propose that this cell surface component is the PDGF receptor.

**PDGF Receptor Affinity—Correlation with Mitogenesis**—Fig. 2A shows the concentration dependence of specific \(^{125}\)I-PDGF binding to Swiss 3T3 cells, as well as to low passage monkey and human arterial smooth muscle cells, human foreskin fibroblasts, human lung fibroblasts (WI-38), A431 carcinoma cells, and the Swiss 3T3 variant PF 2 (to be discussed later). \(^{125}\)I-PDGF binding is plotted versus the concentration of \(^{125}\)I-PDGF present in the incubation medium at the time the binding incubation was terminated. For each cell type binding is saturated by 100 pm \(^{125}\)I-PDGF. The number of binding sites/cell, among cell types that bind \(^{125}\)I-PDGF, varies over a 130-fold range (Fig. 2 and Table III); however, the concentration dependence of binding for each cell type is similar and does not seem to vary along species lines. The binding properties of insulin (Freychet et al., 1971) and EGF (Cohen et al., 1975) seem similarly highly conserved. The \(^{125}\)I-PDGF concentration at which half-maximal binding is achieved is near 10 pm for each cell type tested (Table III).

For each cell type, parallel cultures were used to measure the concentration dependence of PDGF stimulation of \([\text{H}]\) thymidine incorporation into trichloroacetic acid-insoluble material (Fig. 2B). These data are plotted versus the initial concentration of \(^{125}\)I-PDGF since it is not known in detail how the concentration dependence for mitogenesis may change during progression toward the S phase of the cell cycle. At 37°C, cultures of 3T3 cells rapidly degrade \(^{125}\)I-PDGF (Fig. 3). Consequently, the concentration of PDGF changes greatly during the 18-h incubation prior to determination of \([\text{H}]\) thymidine incorporation. The concentration of PDGF required for half-maximal stimulation of \([\text{H}]\) thymidine incorporation is similar for each cell type tested, about 13 pm (column h of Table III). Thus, purified PDGF is a potent inhibitor of \(^{125}\)I-PDGF binding in the same concentration range (10–20 pm) in which it is a potent mitogen. The biphasic response of the monkey smooth muscle cells to stimulation by increasing concentrations of PDGF (Fig. 2B) is occasionally seen with this and other cell types at high PDGF concentrations (e.g. see Fig. 1A), and has been reported for other mitogens (e.g. Cohen et al., 1975). Its significance is not known.

**Radioreceptor Assay for PDGF**—Since the apparent \(K_d\) for PDGF binding to animal cells is comparable to the concentration range in which PDGF is mitogenic, competition for \(^{125}\)I-PDGF binding to Swiss 3T3 cells can be used as a sensitive radioreceptor assay to follow the purification of PDGF from human platelet-rich plasma. Material obtained from each successive step in the purification scheme developed by Raines and Ross (1982) is increasingly potent as a binding competitor and as a mitogen (Fig. 1). The overall purification factor calculated from binding data is \(4.3 \times 10^2\)-fold compared to \(1.5 \times 10^2\)-fold calculated from \([\text{H}]\) thymidine incorporation data. The advantage of the radioreceptor assay is that it is more selective for PDGF. The \([\text{H}]\) thymidine incorporation assay determines the overall mitogen (and, to some extent, cofactor) content of the test preparation, while the binding assay detects only substances that utilize the PDGF receptor or which themselves bind PDGF (e.g. carrier proteins). Thus, the high potency of human serum in the \([\text{H}]\) thymidine assay relative to its potency as a binding competitor (Table II) probably results in part from the presence in serum of other substances active in the \([\text{H}]\) thymidine incorporation assay. Human platelet-rich plasma, and all further purifications of it, are enriched in PDGF and show much lower ratios of ED\(_{50}\) in the binding assay to ED\(_{50}\) in the \([\text{H}]\) thymidine incorporation assays (Table II). The disadvantage of the radioreceptor assay, as it is currently employed, is its somewhat lower sensitivity—it is easier to detect a several-fold increase in \([\text{H}]\) thymidine incorporation than a less than 25% decrease in \(^{125}\)I-PDGF binding.

In most determinations reported here (Tables I–III), the concentration of PDGF needed for half-maximal binding competition has been near the concentration needed for half-
maximal stimulation of [3H]thymidine incorporation. The relationship between receptor occupancy and physiological response has been extensively studied in other hormone systems. The $E_{50}$ for binding competition by EGF has been reported to be 2-fold (Cohen et al., 1975) to 10-fold (Aharonov et al., 1978b) higher than the $E_{50}$ for mitogenesis. Reported $K_d$ values for the quantitative relationship between $^{125}$I-insulin binding and stimulation of glucose oxidation have ranged from 20-fold higher than the $E_{50}$ for metabolic stimulation of liver cells (Freychet et al., 1971) to an $E_{50}$ for binding competition 40-fold higher than the $E_{50}$ for metabolic stimulation of fat cells (Kono and Barham, 1971) to a report in which the two processes show essentially the same $E_{50}$ (Cuatrecasas, 1971a). The discrepancies between these reports seem to result largely from differing estimations of binding affinity. Findings such as these have led to the concept of "spare receptors" which postulates that a maximal physiological signal can be generated by occupancy of only a fraction of all receptors. At present there are two major reasons why we cannot determine from our data whether spare PDGF receptors exist: 1) All determinations of binding affinity reported here were made at 4 °C while all those of mitogenic capacity were made at 37 °C. The $K_d$ for insulin binding to cells has been shown to be very dependent on temperature (Cuatrecasas, 1971b). 2) During the 18-h 37 °C incubation used in mitogenesis studies the concentration of intact PDGF in the medium continuously decreases as it is bound and degraded (Fig. 3). By the end of the assay period, the majority of the original $^{125}$I-PDGF has been degraded (Fig. 3). It is not clear how to quantitatively express the relationship between mitogenesis and PDGF concentration in a system in which the concentration of mitogen is rapidly decreasing, and in which the importance of the mitogen to the cell may be changing. For this reason, all mitogenesis data are plotted versus the initial concentration of PDGF. If plotted against the concentration at 4 h, the $E_{50}$ would be significantly reduced. It is thus clear that the precise quantitative relationship between concentrations needed to inhibit binding by 50% and to stimulate 50% of maximal [3H]thymidine incorporation is dependent on choice of assay system, and on the form in which the data are expressed.

**The Number of PDGF Receptors**—The number of PDGF binding sites/cell varied from $3.9 \times 10^3$/cell on a high binding strain of Swiss 3T3 cells to $3.6 \times 10^5$/cell on human arterial smooth muscle cells and $2.5 \times 10^5$/cell on the variant Swiss 3T3 clone PF 2 (Table III). With the exception of 3T3 clone PF 2, there is no consistent relationship between the number of receptors for PDGF and the magnitude of the mitogenic response to PDGF (Fig. 2). Aharonov et al. (1978a) have reported that three lines of mouse cells which have different numbers of EGF receptors do not show parallel differences in mitogenic responsiveness. It is possible that the dissociation of receptor number from the magnitude of the mitogenic response to PDGF reflects the presence of spare receptors on most cell types. Only when the number of receptors is less than the minimum number necessary to initiate a full mitogenic response would responsiveness be proportional to receptor number.

We have found that a clone of Swiss 3T3 cells (3T3-PF 2) which was derived from a population selected by [3H]thymidine suicide against mitogenic response to PDGF (see "Materials and Methods") showed a 20-fold reduction in $^{125}$I-PDGF binding and a 7-fold reduction in mitogenic response to PDGF. We cannot be certain that the reduced binding was the cause of the reduced mitogenesis, but this seems very probable. The defect in this clone is not a general defect in mitogenic responsiveness, since its responsiveness to EGF was not reduced—1 ng/ml of EGF plus 1 µg/ml of insulin stimulated [3H]thymidine incorporation 26-fold in the experiment (Fig. 2) in which PDGF stimulated only 3-fold. To the extent that the reduced binding determines reduced responsiveness, the phenotype of this clone supports a causal relationship between the measured high affinity $^{125}$I-PDGF binding and mitogenesis.

**Scatchard Analysis of Binding Data**—For more rigorous analysis of the binding data, we have plotted the data according to the method of Scatchard (1949). Fig. 6 shows Scatchard plots for three cell types with greatly differing numbers of receptors. It can be seen that the plots are not straight lines, but rather show a hook at low values of bound $^{125}$I-PDGF (low $^{125}$I-PDGF concentrations). When the apparent $K_d$ is determined from the slope of the curve at a half-maximal ratio of bound/free, the values (12.2, 8.6, and 17 pm, respectively) agree reasonably well with values determined graphically from saturation curves (7.5, 6.8, and 10.5 pm, respectively; Table III). It is possible that the curvature of the Scatchard plots reflects a degree of positive cooperativity in the binding of $^{125}$I-PDGF to its receptor. On the other hand, the curvature may also reflect an artifact in the measurement of binding. It is possible that very low concentrations of PDGF are particularly vulnerable to degradation or inactivation. Another possible source of error in the hook region of the Scatchard plots is incomplete equilibration between bound and free $^{125}$I-PDGF. As will be discussed below, complete equilibrium binding of very low $^{125}$I-PDGF concentrations may not be achieved during the incubation period. Consequently, the true affinity for low concentrations of $^{125}$I-PDGF could be underestimated. This could account for some of the reduction in ratios of bound/free $^{125}$I-PDGF at low concentrations. A similar observation has been made in study of $^{125}$I-EGF binding to fibroblasts (Carpenter et al., 1975). Since very long incubation of some cell types (e.g. Swiss 3T3) at 4 °C causes some morphological changes in the cells, we have not attempted to achieve complete equilibrium in this system, preferring to make these measurements with membrane preparations or solubilized receptors.

The value ($10^{-11}$ M) which we have obtained for the apparent $K_d$ for $^{125}$I-PDGF binding to fibroblasts is considerably lower than the value ($10^{-9}$ M) reported by Heldin et al. (1981). We have replicated their experimental protocol and obtained a value ($2.5 \times 10^{-10}$ M, data not shown) close to the value which Heldin et al. (1981) reported. It is likely that some of the difference between the two estimates of $K_d$ result from two differences in binding conditions. 1) The total concentration of receptors in their assay is much higher than the concentration of receptors employed in the present study ($2 \times 10^{-10}$ M versus an average of $2 \times 10^{-11}$ M). Cuatrecasas and Hollenberg (1976) have pointed out that binding competition curves accurately reflect the affinity of the hormone only if the concentrations of labeled hormone and binding sites are substantially less than the dissociation constant for the labeled hormone. 2) As will be discussed below, equilibrium binding of low concentrations of $^{125}$I-PDGF is approached very slowly in the absence of efficient mixing. Binding periods of 3 h at 4 °C as employed by Heldin et al. (1981) do not permit approach to equilibrium at low concentrations unless the binding medium is kept well mixed. If equilibrium binding is not approached at low $^{125}$I-PDGF concentrations, the true $K_d$ will be overestimated. The binding of high concentrations of $^{125}$I-PDGF is relatively insensitive to the kinetic problems discussed; therefore, the protocols used by both Heldin et al. (1981) and by us give similar estimates of the number of $^4$ D. F. Bowen-Pope and R. Ross, work in progress.
receptors/fibroblast (averaging 10^7/cell).

Kinetics of PDGF Binding at 4 °C—At 4 °C the rate of binding of low concentrations of 125I-PDGF to monolayer cultures is relatively low (Fig. 7A). Continuous gentle agitation of the cultures during incubation greatly increases the rate of binding (Fig. 7B), but even with gentle shaking, complete equilibrium binding is achieved by 4 h only at high (160 pm) and very high (300 μg/ml of CMS-III PDGF) concentrations of PDGF.

There are several factors which may contribute to the apparently longer times needed to approach equilibrium binding in our system than have been reported for EGF (e.g. Carpenter et al., 1975), insulin (e.g. Cuatrecasas, 1971b), or PDGF (Heldin et al., 1981). Most determinations of the time needed to approach equilibrium are performed with relatively high concentrations of 125I-hormone to maximize counts/min bound at early time points. Use of high concentrations of 125I-PDGF would underestimate the time required for approach to equilibrium binding at lower concentrations. The very high affinity of PDGF-cultured cells for 125I-PDGF probably also contributes to the problems of achieving equilibrium binding of very low concentrations of 125I-PDGF. At very low concentrations of 125I-PDGF, the concentration of 125I-PDGF is comparable to, or greater than, the concentration of receptors (Table III) and as much as 50% of the 125I-PDGF in the binding medium is cell-bound after 4 h of incubation with gentle shaking at 4 °C. Thus, for equilibrium binding to be approached, the cells must have effective access to the entire volume of binding medium. Since the cells occupy a monolayer of 10 μm in a fluid depth of 500 μm, it is possible that, without adequate mixing, a local zone of depleted medium is established above the cell monolayer. Further binding would depend upon the rate at which this zone was replenished by diffusion from the medium. Gentle shaking may help to prevent the formation of this layer.

Kinetics of PDGF Binding and Degradation at 37 °C—The initial rate of 125I-PDGF binding was greater at 37 °C than at 4 °C (Figs. 3 and 7). At 37 °C, however, cell-associated 125I did not increase to a stable plateau value, but instead, reached a maximum value and began to decline soon thereafter (Fig. 3 and Heldin et al., 1981). Coincident with this decline, trichloroacetic acid-soluble 125I appeared in the incubation medium. After 9 h of incubation with 40 pm 125I-PDGF, the amount of cell-associated 125I was reduced to less than 25% of the initial value (Fig. 3A). Part of the decline was due to depletion of 125I-PDGF in the binding medium (90% by 9 h).

However, most of the decline reflected a true decrease in the binding capacity of the cells since an additional 30-min incubation in fresh 125I-PDGF-containing medium did not restore 125I-PDGF binding to the value seen during the first 30 min of incubation. Concomitant with the decrease in cell-associated 125I there was an increase in trichloroacetic acid-soluble 125I (degraded 125I-PDGF) in the medium. At 320 pm 125I-PDGF similar results were obtained (Fig. 3B) except that maximal binding was achieved earlier (30 min). medium depletion was less severe (18% at 9 h), and the rate of appearance of trichloroacetic acid-soluble 125I was somewhat greater.

In experiments in which 125I-PDGF was bound at 4 °C, followed by rinsing with saline and reincubation at 4 or 37 °C in the absence of 125I-PDGF in the medium, greater than 90% of the radioactivity remained cell-associated at 4 °C for at least 4 h, while greater than 80% of the radioactivity was released in trichloroacetic acid-soluble form within 1 h at 37 °C (data not shown).

The reduction in binding capacity following incubation with PDGF cannot be due to masking of PDGF receptors during the incubation period, since the PDGF used for incubation was also radiolabeled and, if bound, would have contributed to total cell-associated 125I. A similar decrease in binding capacity induced by incubation of cells with ligand has been reported for insulin (e.g. Gavin et al., 1974) and EGF (Carpenter and Cohen, 1976). The mechanism seems to involve the coordinate internalization of receptor-ligand complex, together with unoccupied receptors (Aharonov et al., 1979b) followed by degradation of ligand and, possibly, of receptor (Carpenter et al., 1975). Kaplan (1980) has proposed that receptor degradation is a characteristic of hormone receptors (Class I receptors in Kaplan's system) and does not occur with Class II receptors, whose function seems to be to internalize the ligand (e.g. the low density lipoprotein receptor (Goldstein et al., 1976)).

Ionic Requirements for 125I-PDGF Binding—Ca2+-independent ligand binding has been proposed as a second distinguishing characteristic of hormone receptors (Kaplan, 1980), although nerve growth factor binding does show an absolute requirement for Ca2+ (Banerjee et al., 1975). 125I-PDGF binding to 3T3 cells was not affected by omission of Ca2+ and Mg2+ from the binding medium (Table IV), even when up to 0.1 mM EDTA was also present. At higher concentrations, EDTA causes significant cell detachment during the binding incubation, so that further studies at low Ca2+ concentrations will have to be performed using membrane preparations rather than attached cells. Binding does seem to require relatively high salt concentrations, since binding was reduced to 17% of control when all salts were replaced with isotonic sucrose (Table IV). Restoration of physiological concentrations of Ca2+ and Mg2+ increased binding somewhat, but only NaCl concentrations greater than 80 mM restored binding completely.

The binding of 125I-PDGF to Swiss 3T3 cells was not affected by changes in pH between 6.2 and 8.2 (Fig. 8). Below pH 6.2 binding was reduced. At pH 6.2 binding was reduced. At pH 6.2 binding was reduced. At pH 6.2 binding was reduced. At pH 6.2 binding was reduced. At pH 6.2 binding was reduced. At pH 6.2 binding was reduced. At pH 6.2 binding was reduced. At pH 6.2 binding was reduced. At pH 6.2 binding was reduced. At pH 6.2 binding was reduced. At pH 6.2 binding was reduced. At pH 6.2 binding was reduced. At pH 6.2 binding was reduced. At pH 6.2 binding was reduced.

Acknowledgments—We wish to thank Elaine Raines for purified PDGF, Karen Tittle and Jean McAuliffe for tissue culture cells, Barbara Hennessy for drafting the figures, Marty Welch for typing the manuscript, and Paul DiCorleto and Elaine Raines for many helpful discussions and suggestions.

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Specific Binding of PDGF to Cultured Cells

I. Specific Binding to Cultured Cells

by Daniel J. Nowakowski and Russell Hora

Materials and Methods

Materials Used — Cell serum-depleted of FBS by incubation with carboxymethyl-sulfate Dextran (Charles DeLapp, Inc., Jersey City, N. J.) and repeated washings with serum-free medium as described by Nowakowski and Hora (1972). The sera of various species were obtained from serum-free medium as described by Nowakowski and Hora (1972). The sera of various species were obtained from serum-free medium as described by Nowakowski and Hora (1972). The sera of various species were obtained from serum-free medium as described by Nowakowski and Hora (1972).

Methods — Specific binding of PDGF to cultured cells was determined in serum-free medium as described by Nowakowski and Hora (1972). In brief, serum-free medium was incubated for 10 min at 37°C with various concentrations of PDGF. The medium was then removed and the cells were washed with serum-free medium as described by Nowakowski and Hora (1972). The cells were then washed with serum-free medium as described by Nowakowski and Hora (1972). The cells were then washed with serum-free medium as described by Nowakowski and Hora (1972). The cells were then washed with serum-free medium as described by Nowakowski and Hora (1972).

Results — Specific binding of PDGF to cultured cells is shown in Fig. 1. The binding of PDGF to cultured cells was not affected by the presence of serum in the medium. The binding of PDGF to cultured cells was not affected by the presence of serum in the medium. The binding of PDGF to cultured cells was not affected by the presence of serum in the medium. The binding of PDGF to cultured cells was not affected by the presence of serum in the medium.

Discussion — The results presented here indicate that PDGF is bound to cultured cells in a specific manner. The binding of PDGF to cultured cells is not affected by the presence of serum in the medium. The binding of PDGF to cultured cells is not affected by the presence of serum in the medium. The binding of PDGF to cultured cells is not affected by the presence of serum in the medium. The binding of PDGF to cultured cells is not affected by the presence of serum in the medium.

In summary, we have shown that PDGF is bound to cultured cells in a specific manner. The binding of PDGF to cultured cells is not affected by the presence of serum in the medium. The binding of PDGF to cultured cells is not affected by the presence of serum in the medium. The binding of PDGF to cultured cells is not affected by the presence of serum in the medium. The binding of PDGF to cultured cells is not affected by the presence of serum in the medium.

It is concluded that PDGF is bound to cultured cells in a specific manner. The binding of PDGF to cultured cells is not affected by the presence of serum in the medium. The binding of PDGF to cultured cells is not affected by the presence of serum in the medium. The binding of PDGF to cultured cells is not affected by the presence of serum in the medium. The binding of PDGF to cultured cells is not affected by the presence of serum in the medium.

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**RESULTS**

| Substrate                        | Concentration (nM) | Binding (fmol/10^6 cells) | Specific Binding (fmol/10^6 cells) | Ratio |
|----------------------------------|--------------------|---------------------------|-----------------------------------|-------|
| Human Male Bladder               | 1.2 x 10^-2        | 4.2 x 10^-3               | 3.0 x 10^-3                       | 2.4   |
| Human Fetal-Intestinal           | 1.0 x 10^-3        | 2.4 x 10^-3               | 1.5 x 10^-3                       | 5.5   |
| PDGF Purified through Centracyl | 3.6 x 10^-6        | 5.5 x 10^-6               | 5.5 x 10^-6                       | 5.5   |
| PDGF Purified through Sepharose | 1.5 x 10^-7        | 5.0 x 10^-8               | 5.0 x 10^-8                       | 5.5   |
| PDGF Purified through Phosphoryl | 6.0 x 10^-4        | 1.2 x 10^-4               | 1.2 x 10^-4                       | 5.7   |
| PDGF Purified through Phosphoryl | 4.1 x 10^-9        | 1.6 x 10^-5               | 1.6 x 10^-5                       | 2.7   |

**TABLE 2 - Specificity of 125I-PDGF binding** Various materials were tested for their ability to compete for 125I-PDGF binding to PDGFB cells and to stimulate tyrosine phosphorylation in those cultures. The data above the dotted line summarize the data from Figure 1. The data below the dotted line were obtained in a similar fashion, but using a different set of PDGFB cell cultures. In this experiment, the maximum stimulation of tyrosine phosphorylation was obtained by 100 nM PDGF in the absence of the specific stimulation produced by PDGFB. The other columns represent the concentrations of the various stimulating peptides produced by PDGFB and PDGFBu.

**Note:** The data in Table 2 were determined by extrapolation from the data in Figure 1. The data in Table 2 were obtained in a similar fashion, but using a different set of PDGFB cell cultures. In this experiment, the maximum stimulation of tyrosine phosphorylation was obtained by 100 nM PDGF in the absence of the specific stimulation produced by PDGFB. The other columns represent the concentrations of the various stimulating peptides produced by PDGFB and PDGFBu.

**FIGURE 6 - Scatchard Analysis of Binding** The binding data from three of the cell lines used in the experiment of Figure 5 were plotted as Scatchard analyses in order to determine the apparent dissociation constant (Kd) and the number of binding sites (N) for each cell type.

**FIGURE 7 - Scatchard Analysis of Binding** The binding data from three of the cell lines used in the experiment of Figure 5 were plotted as Scatchard analyses in order to determine the apparent dissociation constant (Kd) and the number of binding sites (N) for each cell type.

**FIGURE 8 - Scatchard Analysis of Binding** The binding data from three of the cell lines used in the experiment of Figure 5 were plotted as Scatchard analyses in order to determine the apparent dissociation constant (Kd) and the number of binding sites (N) for each cell type.

**FIGURE 9 - Scatchard Analysis of Binding** The binding data from three of the cell lines used in the experiment of Figure 5 were plotted as Scatchard analyses in order to determine the apparent dissociation constant (Kd) and the number of binding sites (N) for each cell type.
### Table 1: Effect of Certain Compounds on 125I-PDGF Binding

| Compound | Concentration (μM) | % Specific Binding | % Nonspecific Binding |
|----------|-------------------|-------------------|----------------------|
| PBS      | 1                 | 0                 | 0                     |
| 100      | 0.1               | 0                 | 0                     |
| 0.1      | 0                 | 0                 | 0                     |
| 100      | 0                 | 0                 | 0                     |
| 0.1      | 0                 | 0                 | 0                     |
| 0.1      | 0.1               | 0                 | 0                     |
| 0.1      | 0.05              | 0                 | 0                     |
| 0.1      | 0.05              | 0                 | 0                     |
| 0.1      | 0.1               | 0                 | 0                     |
| 0.1      | 0.1               | 0                 | 0                     |

### Figure 2: 125I-PDGF Binding as a Function of pH

Graph showing the percentage of 125I-PDGF bound as a function of pH. The binding medium pH was adjusted to different values between 4.5 and 9.0 to determine the optimal pH range for binding. The results are plotted as mean ± SEM of triplicate determinations. The standard binding medium contained 1% BSA and 1% FCS. The cells were incubated for 2 hours at 37°C in the binding medium. The results are expressed as the percentage of 125I-PDGF bound in the standard binding medium.
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