Interactions between the Receptors for Platelet-derived Growth Factor and Epidermal Growth Factor

DANIEL F. BOWEN-POPE, PAUL E. DICORLETO, and RUSSELL ROSS
Departments of Biochemistry and Pathology, University of Washington, Seattle, Washington 98195. Dr. DiCorleto's present address is the Division of Research, Cleveland Clinic Foundation, Cleveland, Ohio 44106.

ABSTRACT Preincubation of Swiss 3T3 cells or human fibroblasts with purified platelet-derived growth factor (PDGF) at 4°C or 37°C rapidly inhibits subsequent binding of ^{125}I-epidermal growth factor (^{125}I-EGF). The effect does not result from competition by PDGF for binding to the EGF receptor since (*a*) very low concentrations of PDGF are effective, (*b*) cells with EGF receptors but no PDGF receptors are not affected, and (*c*) the inhibition persists even if the bound PDGF is eluted before incubating the cells with ^{125}I-EGF. PDGF does not affect ^{125}I-insulin binding nor does EGF affect ^{125}I-PDGF binding under these conditions. Endothelial cell-derived growth factor also competes for binding to PDGF receptors and inhibits ^{125}I-EGF binding. The inhibition demonstrated by PDGF seems to result from an increase in the K_d for ^{125}I-EGF binding with no change in the number of EGF receptors.

A number of substances have been shown to affect ^{125}I-EGF binding to cells after a period of preincubation at 37°C, including dexamethasone (1), fibroblast-derived growth factor (2), vasopressin (3), and lipid-interacting substances (4). Lee and Weinstein (5) found that different phorbol esters could inhibit ^{125}I-EGF binding in proportion to their potency as tumor promoters. The effect is temperature-dependent (3, 6, 7, 8) and seems to reflect a decrease in the affinity of the EGF receptor (3), possibly by eliminating a high affinity class of EGF receptors (8). Phorbol acetate does not seem to affect ^{125}I-insulin binding (7). Phorbol esters seem to have specific binding sites on responsive cells (9, 10), so it is possible that their effect on EGF binding is mediated through a specific receptor.

Recently, incubation with PDGF at 37°C has been shown to decrease the binding of ^{125}I-EGF (11, 12, 13), reportedly by reducing the number of EGF receptors (11, 13). PDGF is the major mitogen in serum for many connective tissue cells in culture (14, 15, 16). At 37°C cells respond rapidly to PDGF with changes in several processes that could have secondary effects on receptors for EGF and other growth factors. PDGF stimulates the phosphorylation of cytoplasmic proteins on tyrosine within 1 min (17), increases phosphatidylinositol turnover within 2 min (18), phosphorylation of a 33-dalton cell protein within 3 min (19), the appearance of membrane ruffles and microvilli within 20 min (20), and increased amino acid uptake within 1 h (21). Over a period of several hours, PDGF increases the number of LDL receptors (22, 23) and somatomedin C receptors (24). Serum stimulates ^{86}Rb+ influx within 2 min (26), hexose uptake within 10 min (25), and phosphofructokinase activity within 30 min (27). This series of complex responses makes it difficult to distinguish direct interaction between PDGF and EGF receptors from effects which are secondary to the metabolic effects of PDGF. In an attempt to reduce the possibilities for secondary effects of PDGF on EGF receptors, we have investigated the effects of PDGF and other hormones on ^{125}I-EGF binding in a system in which both pretreatment and binding determinations are performed at 4°C.

MATERIALS AND METHODS

The Swiss mouse 3T3-D1 cells used in most of the studies reported here were cloned from Swiss 3T3 cells obtained from R. Pollack (State University of New York at Stony Brook). The A-431 human carcinoma cells were obtained from B. Gallis (University of Washington, Seattle). The adult human diploid foreskin fibroblasts (HF) were obtained essentially as described by Baker et al. (1). The variant 3T3-PF2 cell was selected by [^{3}H]thymidine suicide against mitogenic response to PDGF, as described in Bowen-Pope and Ross (28), and recloned for these experiments (now designated 3T3-PF2 (f)). Culture methods were those previously described (28). Binding of ^{125}I-ligand was initiated, as described in the figure and table legends, either by adding 50 μl of ^{125}I-ligand directly to the preincubation medium or by rinsing the cultures and incubating with fresh binding medium containing ^{125}I-ligand alone. The methods for determining specific binding were those previously described (28). Nonspecific binding of ^{125}I-PDGF and ^{125}I-EGF averaged 1–3% and 5–15%, respectively, of total binding. PDGF was radioiodinated to a specific activity of about 40,000 cpm/ng, essentially as previously described (28). EDGF was prepared from serum-free conditioned medium of confluent bovine aortic endothelial cells as previously described (29). Briefly, the medium was centrifuged for 6×10^6 *g*-minutes, concentrated 30-fold by ultrafiltration (final protein concentration 655 μg/ml), and dialyzed

exhaustively against 1 mM ammonium bicarbonate. ^{125}I-insulin and ^{125}I-EGF were obtained from New England Nuclear (Boston, MA). Highly purified human PDGF was prepared as previously described (30). EGF and FGF were obtained from Collaborative Research (Waltham, MA) and porcine insulin from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Reduction of ^{125}I-EGF Binding by Preincubation with PDGF at 4 °C

Fig. 1 *a* shows the effect of incubation of 3T3 cells at 4°C with PDGF on subsequent binding of ^{125}I-PDGF or ^{125}I-EGF. As expected, ^{125}I-PDGF binding is reduced by competition with unlabeled PDGF for the PDGF receptor (28, 31). Unexpectedly, the preincubation with PDGF also reduces subsequent ^{125}I-EGF binding (Fig 1 *a*), even though all incubations were performed at 4°C to prevent internalization of bound ligand (32, 33, 34) and possible cointernalization of PDGF and EGF receptors. Inhibition of ^{125}I-EGF binding is detectable with concentrations of PDGF too low to produce measurable competition for ^{125}I-PDGF binding under these conditions. The maximal effect of PDGF in this experiment is a 60% decrease in ^{125}I-EGF binding at 2 ng/ml PDGF, with no further decrease occurring at higher concentrations of PDGF. The converse effect is not seen, i.e., preincubation with EGF at concentrations sufficient to saturate the EGF receptor does not affect ^{125}I-PDGF binding (Fig. 1 *b*). The small reduction in ^{125}I-PDGF binding seen in this experiment was not reproducible.

Mediation through PDGF Receptors

The concentration dependence of PDGF inhibition of ^{125}I-EGF binding suggested that the effect is mediated through PDGF receptors and not through a direct effect of PDGF on EGF receptors. To look for direct effects we used A-431 cells, which have EGF receptors but not PDGF receptors (28, 31, 35). Even extremely high concentrations of PDGF (120 ng/ml) do not reduce ^{125}I-EGF binding to A-431 cells (data not shown). We have also selected a variant line of 3T3 cells (3T3-PF2 (f)) that shows a greatly reduced mitogenic response to PDGF and expresses only ~5% of the parental number of PDGF receptors per cell (28), but that expressed the parental number of EGF receptors. Fig 2 shows that PDGF reduces ^{125}I-EGF binding to these cells, but to a much smaller extent than it does to normal 3T3 cells. This suggests that the inhibition of ^{125}I-EGF binding by PDGF is proportional to the number of PDGF receptors present. However, until the biochemical nature of the defect in clone PF2(f) is known, we cannot use this cell line to make any exact calculations as to the relationship between occupied PDGF receptors and inhibition of EGF binding. Inhibition of ^{125}I-EGF binding by PDGF is not unique to 3T3 cells since binding of ^{125}I-EGF to diploid human fibroblasts, which express both PDGF and EGF receptors (28, 31, 32), is also reduced by preincubation at 4°C with PDGF (Fig. 2).

Simultaneous Occupation of the PDGF Receptors is Not Required

In the previous experiments PDGF was present prior to, and during, incubation with ^{125}I-EGF. To determine whether the inhibition of ^{125}I-EGF binding requires continuous occupation of the PDGF receptors by PDGF during the period of ^{125}I-EGF binding, we removed nonbound PDGF with PBS rinses

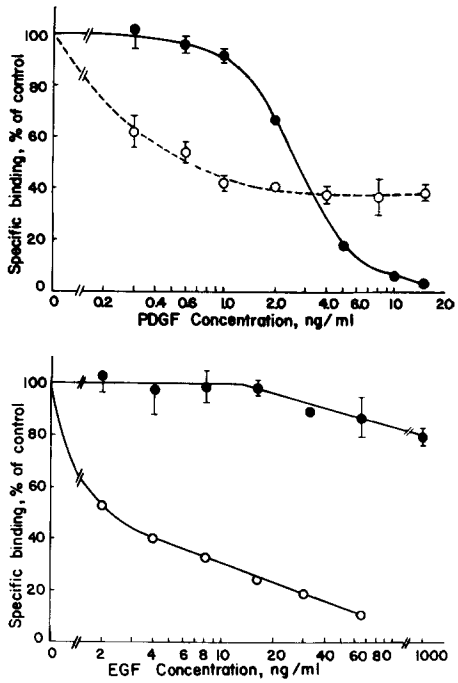

FIGURE 1 Effect of preincubation with PDGF or EGF on subsequent ^{125}I-PDGF and ^{125}I-EGF binding. Confluent quiescent cultures of 3T3-D1 cells were rinsed with cold PBS and incubated 2 h at 4°C with 1.0 ml per well of binding medium containing the concentrations of PDGF (*a*) or EGF (*b*) indicated on the abscissa. 50 μl of iodinated ligand was then added to give 0.2 ng/ml ^{125}I-PDGF (●) or 0.25 ng/ml ^{125}I-EGF (○) and incubation was continued for 2 h at 4°C. Specific binding (means ± SEM of determinations on triplicate cultures) was measured as described in Materials and Methods, and is plotted as % of specific binding in binding medium alone.

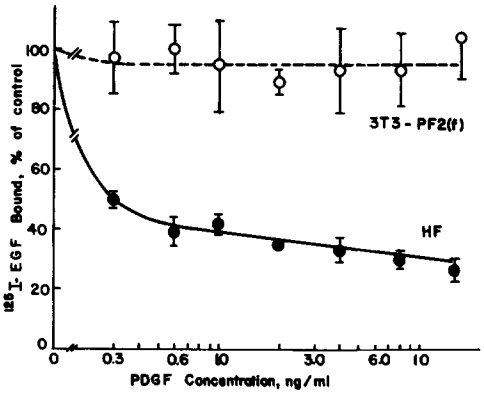

FIGURE 2 Effect of PDGF and EGF on ^{125}I-EGF binding to human fibroblasts and PDGF receptor deficient 3T3 variants. Confluent quiescent cultures of adult human foreskin fibroblasts (●) or of 3T3-PF2(f) cells (○ were incubated for 2 h at 4°C in binding medium with the concentration of PDGF indicated on the abscissa). 50 μl of ^{125}I-EGF was added to give 0.25 ng/ml ^{125}I-EGF and the incubation was continued for 1 h. Specific ^{125}I-EGF binding (mean ± SEM of determinations on triplicate cultures) was determined as described in Materials and Methods, and is plotted as % of binding in the absence of PDGF.

before measuring ^{125}I-EGF binding. Binding of ^{125}I-EGF was still inhibited. However, PDGF dissociates so slowly from its receptor at 4°C (31) that most receptors occupied during the preincubation period would remain occupied during the incubation with ^{125}I-EGF. To remove bound PDGF, we adapted the method of Haigler et al. (36) to remove cell surface-bound

^{125}I-EGF. A 3-min incubation at 4°C with 0.1% acetic acid in 150 mM NaCl with 0.1% BSA removed 89% of prebound ^{125}I-PDGF and 78% of prebound ^{125}I-EGF (data not shown). Neither the 4°C preincubation nor the acetic acid rinse damaged the cells or receptors as measured by three criteria (*a*) <2% of the cells stained with the dye trypan blue, even after a 4-h incubation at 4°C in binding medium followed by an acetic acid rinse. (*b*) Binding of ^{125}I-EGF and ^{125}I-PDGF to acid-treated cultures was 101% and 103%, respectively, of binding to untreated cultures. (*c*) Cultures of 3T3 cells incubated in binding medium for 4 h at 4°C, then rinsed with acetic acid, were able to respond normally to subsequent addition of 5 ng/ml EGF (3.7-fold stimulation in control, 3.2-fold stimulation in treated cultures) or PDGF (5.1-fold stimulation in control, 4.4-fold stimulation in treated cultures) as assayed by [^{3}H]-thymidine incorporation determined 20 h later.

Table I shows that the acid rinse eliminated the effects of preincubation with PDGF on subsequent ^{125}I-PDGF binding, presumably by releasing prebound ligand, but did not significantly lessen the inhibition of subsequent ^{125}I-EGF binding. This demonstrates that PDGF is not acting by sterically blocking ^{125}I-EGF binding and that the effect of PDGF is stable for at least the time needed to assay ^{125}I-EGF binding.

Kinetics of Inhibition

At 4°C the effect of PDGF on ^{125}I-EGF binding is rapid but not immediate (Fig. 3). A portion of the time (about 10 min) needed to produce a 50% decrease in subsequent ^{125}I-EGF binding probably reflects the time needed for binding of PDGF to its receptor (e.g., see reference 28 for binding kinetics under these conditions). However, some time after binding is also required to develop the full effect, since the inhibition produced by short preincubation with PDGF is considerably less when the bound PDGF is removed with acetic acid before assaying ^{125}I-EGF binding, than when the PDGF remains bound throughout the assay period (Fig. 3). This cannot be due to instability of the inhibition once established, since dissociation of bound PDGF has little effect after a preincubation of 2 h or more (Table I and Fig. 3).

TABLE I

Effect of Preincubation with Mitogen on Subsequent Binding of Labeled Mitogen

Test substance during preincubation	^{125}I-ligand	^{125}I-ligand binding % of control*	
		PBS rinse	Acetic acid rinse
0	^{125}I-PDGF	100	100
10 ng/ml PDGF		17 ± 4	101 + 4
100 ng/ml EGF		105 ± 4	99 ± 4
200 ng/ml FGF		100 ± 3	98 ± 5
200 ng/ml insulin		107 ± 5	98 ± 2
100 μl/ml EDGF		83 ± 3	93 ± 2
0	^{125}I-EGF	100	100
10 ng/ml PDGF		18 ± 3	28 ± 8
100 ng/ml EGF		38 ± 2	128 ± 13
200 ng/ml FGF		67 ± 2	78 ± 6
200 ng/ml insulin		111 ± 4	96 ± 4
100 μl/ml EDGF		35 ± 2	34 ± 2

Confluent cultures of 3T3 cells were preincubated for 2 h at 4°C in binding medium with the indicated test substance. The cultures were then rinsed three times with binding rinse and incubated for 3 min at 4°C with either PBS or 0.1% acetic acid in 150 mM NaCl with 0.1% BSA. These rinse solutions were aspirated and the cultures incubated for 30 min at 4°C with 1 ml per well of binding medium containing 0.2 ng/ml ^{125}I-PDGF or 0.25 ng/ml ^{125}I-EGF.

* Specific binding was determined as described in Materials and Methods. Specific binding (means ± SEM of determinations on triplicate cultures) is expressed as % of the value obtained after preincubation without test addition.

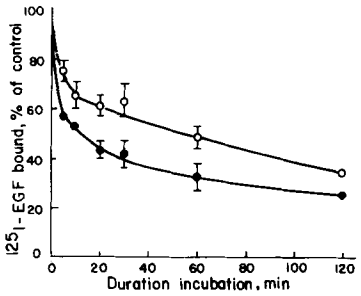

FIGURE 3 Time course of effect of PDGF on subsequent ^{125}I-EGF binding. Confluent quiescent cultures of 3T3 D1 cells were rinsed with cold PBS and incubated at 4°C in binding medium with 10 ng/ml PDGF. After the times indicated on the abscissa the cultures were rinsed twice with PBS and incubated for 3 min at 4°C with either PBS (●) or 0.1% acetic acid in 150 mM NaCl with 0.5% BSA (○). These solutions were aspirated and the cultures incubated for 1 h at 4°C in binding medium with 0.25 ng/ml ^{125}I-EGF. Specific binding (mean ± SEM of determinations on triplicate cultures) was determined as described in Materials and Methods, and is plotted as % of the value obtained without exposure to PDGF.

Specificity of Inhibition of EGF Binding

Table I shows that PDGF is able to inhibit ^{125}I-EGF binding. The inhibition by unlabeled EGF presumably represents steric competition for EGF receptors, since it is eliminated by removing prebound EGF with acetic acid. Binding of ^{125}I-EGF is also inhibited by a factor produced by cultured vascular endothelial cells in serum-free medium (Table I). Since endothelial cell-derived growth factor (ECDGF) does not block binding of ^{125}I-EGF to cells without PDGF receptors (unpublished observations), and since ECDGF seems to bind to PDGF receptors (28), it seems likely that the inhibition of ^{125}I-EGF binding by ECDGF is also mediated through the PDGF receptor. In three experiments, fibroblast growth factor (FGF) had a smaller, though statistically significant, effect on ^{125}I-EGF binding under these conditions (Table I). Although the preparation of FGF is estimated to be only 10% pure, it is unlikely that contamination by EGF is responsible for the inhibition of ^{125}I-EGF binding since the inhibition was not reversed by acetic acid rinsing. Significant contamination by PDGF is also unlikely since ^{125}I-PDGF binding is not significantly reduced by 10 μg/ml FGF (28). High concentrations of insulin had no effect (Table I). The converse interaction, i.e., inhibition of ^{125}I-PDGF binding by preincubation with EGF, does not occur (Fig. 1*b* and Table I).

To investigate the specificity of PDGF inhibition of ligand binding we investigated the sensitivity of ^{125}I-insulin binding to inhibition by PDGF. Even extremely high concentrations of PDGF (400 ng/ml) did not reduce specific ^{125}I-insulin binding to 3T3 cells (data not shown).

Scatchard Analysis

Fig. 4*a* shows that preincubation of 3T3 cell cultures with PDGF at 4°C decreases ^{125}I-EGF binding by increasing the apparent dissociation constant with little effect on the apparent number of EGF receptors, and that the magnitude of the effect depends on the concentration of PDGF used. A simple K_d

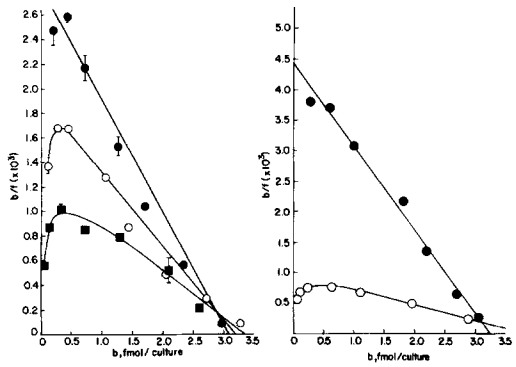

FIGURE 4 Scatchard analysis of ^{125}I-EGF binding. (*a*) Confluent quiescent cultures of 3T3-D1 were preincubated for 2 h at 4°C in binding medium without PDGF (●), with 0.5 mg/ml PDGF (○), or with 10 ng/ml PDGF (■). Preincubation was terminated by rinsing with PBS and was followed by incubation for 2 h at 4°C in binding medium with concentrations of ^{125}I-EGF from 0.5 to 225 ng/ml. Specific ^{125}I-EGF binding (mean ± SEM of determinations on triplicate cultures) was determined as described in Materials and Methods. (*b*) Cultures were preincubated for 2 h at 37°C in bicarbonate-buffered medium without PDGF (●) or with 10 ng/ml PDGF (○). Subsequent analysis was as described for (*a*).

cannot be calculated for PDGF-preincubated cultures due to the pronounced curvature of the plot at low concentrations of EGF. However, from binding saturation plots we calculate that cultures preincubated with 0, 0.5, or 10 ng/ml PDGF show half-maximal ^{125}I-EGF binding at 1.3, 1.6, and 2.0 nM, respectively, and express 40,200, 41,700, and 43,800 EGF receptors/cell, respectively. After pretreatment with either concentration of PDGF, the maximum ratio of bound to free ^{125}I-EGF occurs when ~12% of the EGF receptors are occupied. Fig 4*b* shows that preincubation of 3T3 cell cultures with 10 ng/ml PDGF at 37°C for 2 h causes a similar, though more pronounced, decrease in the apparent K_d of ^{125}I-EGF binding with no significant decrease in the apparent number of EGF receptors.

DISCUSSION

We have found that PDGF can reduce the binding of low concentrations of ^{125}I-EGF by up to 80%, even when the cells are maintained at 4°C throughout the preincubation and measurement periods. This distinguishes PDGF from other inhibitors of ^{125}I-EGF binding that have been shown to require a period of incubation at 37°C to become effective (1, 3, 6, 7, 8, 32, 34). We have demonstrated that inhibition does not result from direct competition by PDGF for ^{125}I-EGF binding, nor from a general inhibition of specific ligand binding. These findings suggest that caution must be used in interpreting the results of "competition" studies, including radioreceptor assays performed using living cells, since there seems to be significant interaction between the two different receptor systems, even at 4°C.

We can only speculate on the mechanism through which PDGF is affecting the EGF receptor. The process seems to be "catalytic" rather than stoichiometric in that occupation of the relatively small number of PDGF receptors produces half maximal inhibition of ^{125}I-EGF binding. Internalization of EGF receptors in response to PDGF binding to its own receptor ("cointernalization") does not seem to be involved, since PDGF can inhibit ^{125}I-EGF binding even at 4°C and since the affinity, but not the number, of EGF receptors is reduced. Among the ligand-induced changes that are not prevented by incubation at 4°C, two processes appear to be relatively good candidates for involvement in the interaction between the two receptor systems—stimulation of protein kinase activity and clustering of receptors.

Although most energy-dependent reactions are greatly reduced at 4°C, the activity of the EGF- and PDGF-stimulated, tyrosine-specific protein kinases in membrane preparations is still substantial at 4°C (37, 38, 39, and our unpublished observations). In each case, one of the substrates for the kinase seems to be the receptor for the activating ligand (39, 40). It is possible that binding of PDGF to its receptor stimulates phosphorylation of the EGF receptor as well as of its own receptor. Unfortunately, we have not yet been able to resolve phosphorylated PDGF and EGF receptors on SDS polyacrylamide gels due to their very similar size (150,000–170,000 daltons) (39, 40, 41, 42, 43). The effect of phosphorylation on the properties of the receptors is not known. However, phosphorylation-dephosphorylation reactions have been shown to affect the enzymatic activities of several regulatory enzymes (reviewed in reference 44). The effect of phosphorylation on enzymes can be to change the K_m for a substrate, the K_a for an activator, or the K_i for an inhibitor (44). If PDGF-stimulated phosphorylation of the EGF receptor were involved in the inhibition of ^{125}I-EGF binding, the site(s) of phosphorylation may be different from the site(s) phosphorylated in response to EGF binding, since removal of EGF (but not of PDGF) restores the binding properties of the EGF receptor. Hunter and Cooper (45) and Gates and King (46) have reported that the EGF receptor can be phosphorylated at multiple sites. Possibly PDGF stimulates phosphorylation of a very stable site.

Another candidate for involvement in the PDGF inhibition of EGF binding is an induced aggregation of EGF receptors with PDGF receptors. Some receptors seem to be present in small patches even before ligand addition. Low density lipoprotein (LDL) receptors are largely preconcentrated in coated pit regions (47). Large-scale patching and internalization of receptors does not occur at 4°C (33, 34, 36). Nevertheless, several receptors have been shown to be mobile in the plasma membrane to 4°C (48, 49) and to form small aggregates in response to ligand binding (48). It is possible that the inclusion of an EGF receptor in the same aggregate as PDGF receptors could influence its ability to bind ^{125}I-EGF. Certainly, receptors are sensitive to general changes in their local environment. Phospholipase C and other lipid-interactive substances seem to decrease the affinity of ^{125}I-EGF binding to mink lung cells (4), and removal of the EGF receptor from the membrane into Triton micelles reduces its affinity for EGF by 10-fold (50). Changes in other membrane proteins associated with receptors have also been implicated in changes in receptor properties. Maturo and Hollenberg (51) reported a membrane glycoprotein whose association with the solubilized, partially purified insulin receptor altered its apparent affinity for ^{125}I-insulin.

It is intriguing to speculate on the possible significance of this phenomenon for cell behavior. Dicker and Rozengurt (52) reported that phorbol acetate and EGF had synergistic effects in stimulating DNA synthesis, despite the fact that phorbol acetate reduces ^{125}I-EGF binding. Wolfe et al., (53) reported that EGF does not down-regulate its own receptor if HeLa cells are cultured in defined medium without serum. PDGF may represent the component in serum which is necessary for the down-regulation of the EGF receptor in this system. It is thus possible that the inhibition of ^{125}I-EGF binding by PDGF that we have described in this report is only one manifestation of the interaction between the PDGF and EGF receptors, i.e.,

PDGF may also alter the ability of the EGF receptors to affect intracellular events or to be internalized and degraded. In any case, the ability of one growth factor to alter the properties of receptors for a second growth factor adds another locus to the sites at which the different polypeptide hormones could interact to potentiate or inhibit their physiological effects.

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Note Added in Proof: We have recently reevaluated the PDGF content of our purified PDGF using determinations of protein content by OD_{280}, Lowry assay, and quantification of silver-stained SDS PAGE; and using determinations of purity by silver-stained SDS PAGE and determination of the fraction of ^{125}I-PDGF able to bind specifically to PDGF-responsive cells. On the basis of this new information we have concluded that the values for PDGF concentrations reported in this paper should be multiplied by 0.28 to obtain the corrected values.

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