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

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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 125I-epidermal growth factor (125I-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 125I-EGF. PDGF does not affect 125I-insulin binding nor does EGF affect 125I-PDGF binding under these conditions. Endothelial cell-derived growth factor also competes for binding to PDGF receptors and inhibits 125I-EGF binding. The inhibition demonstrated by PDGF seems to result from an increase in the Kd for 125I-EGF binding with no change in the number of EGF receptors.

A number of substances have been shown to affect 125I-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 125I-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 125I-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 125I-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-kilodalton 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 46Rb+ 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 125I-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 [3H]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 125I-ligand was initiated, as described in the figure and table legends, either by adding 50 μl of 125I-ligand directly to the preincubation medium or by rinsing the cultures and incubating with fresh binding medium containing 125I-ligand alone. The methods for determining specific binding were those previously described (28). Nonspecific binding of 125I-PDGF and 125I-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). PDGF 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...
RESULTS

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

Fig. 1a 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. 1a), 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. 1b). 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 (28, 31, 32) which have EGF receptors but not PDGF receptors (28, 31, 32). 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 (28, 31, 32). 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 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...
**TABLE I**

| Test substance during preincubation | 
|-------------------------------------|
| 125I-ligand binding % of control* |
| 125I-ligand binding                   |
| PBS rinse                            |
| Acetic acid rinse                    |
| 0                                   |
| 10 ng/ml PDGF                       |
| 100 ng/ml EGF                       |
| 200 ng/ml FGF                       |
| 100 μl/ml insulin                   |
| 0                                   |
| 10 ng/ml PDGF                       |
| 100 ng/ml EGF                       |
| 200 ng/ml FGF                       |
| 100 μl/ml insulin                   |
| 0                                   |
| 10 ng/ml PDGF                       |
| 100 ng/ml EGF                       |
| 200 ng/ml FGF                       |
| 100 μl/ml insulin                   |
| 0                                   |
| 10 ng/ml PDGF                       |
| 100 ng/ml EGF                       |
| 200 ng/ml FGF                       |
| 100 μl/ml insulin                   |

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 3 min at 4°C with 1 ml per well of binding medium containing 0.2 ng/ml 125I-PDGF or 0.25 ng/ml 125I-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.

Specificity of Inhibition of EGF Binding

Table I shows that PDGF is able to inhibit 125I-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 125I-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 (ECDFG) does not bind to PDGF receptors (28), it seems likely that the inhibition of 125I-EGF binding by ECDFG is also mediated through the PDGF receptor.

Scatchard Analysis

Fig. 4a shows that preincubation of 3T3 cell cultures with PDGF at 4°C decreases 125I-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 Kd...
Among the ligand-induced changes that are not prevented by 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 $K_a$ for a substrate, the $K_\text{cat}$ 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 125I-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 125I-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 125I-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 125I-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 125I-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 125I-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 OD280, 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 125I-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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