Evidence for Functional Platelet-derived Growth Factor Receptors on MG-63 Human Osteosarcoma Cells

Dana T. Graves, Harry N. Antoniades, Steven R. Williams, and Albert J. Owen

ABSTRACT

The specific interaction of platelet-derived growth factor (PDGF) with the human osteosarcoma cell line MG-63 was studied. Scatchard analysis of $^{125}$I-PDGF binding to MG-63 cells indicated there were 32,000 specific PDGF-binding sites per cell with a $K_d$ of $2.4 \times 10^{-11}$ M. Unlabeled PDGF blocked the specific binding of labeled PDGF to MG-63 cells at concentrations greater than 1 ng/ml. When assayed for phosphorylation of MG-63 membrane vesicles, PDGF was shown to stimulate a dose-dependent phosphorylation of a protein (phosphoprotein with a molecular weight of 185,000) which was stable in 1 M NaOH. In the absence of PDGF, a prominent alkali-stable phosphoprotein was noted. When tested for secretion of PDGF-like factors, the mitogenic activity of MG-63-conditioned serum-free medium was not blocked by anti-PDGF antiserum. Concentrated MG-63-conditioned medium did not compete with $^{125}$I-PDGF for specific receptor sites on diploid fibroblasts. Therefore, MG-63 osteosarcoma cells have functional PDGF receptors and do not secrete PDGF-like mitogens.

INTRODUCTION

It has previously been reported that the U-2 OS (formerly 2T) osteosarcoma cell line secretes a PDGF-like mitogen (11, 12), and does not respond to exogenous PDGF (11). Nontransformed mesenchymal cells require both PDGF and factors present in PPP for growth (19), while transformed cells, such as the U-2 OS, may overcome the PDGF requirement. The U-2 OS osteosarcoma cells apparently satisfy their requirement for PDGF by endogenous secretion, which is consistent with the autocrine hypothesis (22). The importance of these findings was demonstrated by recent reports that the oncogene in the simian sarcoma virus, v-sis, is capable of coding for PDGF, suggesting that PDGF production plays an important role in the transformation process (9, 23).

In collaboration with researchers at the California Institute of Technology, we have recently established that mRNA from the U-2 OS cells hybridizes with cDNA probes based on the v-sis probe. It appears, therefore, that transformation of the U-2 OS cells involves activation of sis-gene transcription resulting in the production of biologically active PDGF. This process however, may not be universal in the development of osteogenic sarcomas.

In this paper we present evidence that proliferation of MG-63 osteosarcoma cells does not involve endogenous secretion of PDGF-like factors. It was found that MG-63 cells produced mitogenic activity which was not PDGF-like, had membrane receptors to PDGF, and responded to exogenous sources of PDGF. When assayed for phosphorylation of membrane proteins, a high degree of apparent tyrosine kinase activity was noted without the addition of PDGF.

MATERIALS AND METHODS

Human osteosarcoma cells (MG-63) and BALB/c-3T3 clone A31 mouse fibroblasts were purchased from the American Type Culture Collection, Rockville, MD. The MG-63 osteosarcoma cell line was originally isolated and characterized by Heremans et al. (14). Human fetal fibroblasts (GM-10) were purchased from the Genetic Mutant Repository, Camden, NJ. Human fibroblasts and osteosarcoma cells were grown in MEM (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). PDGF I and PDGF II (3000 units/g protein) were isolated and separately labeled with $^{125}$I by Iodo-Beads (Pierce Chemical Co., Rockford, IL). Labeled PDGF II was used in binding experiments. Partially purified PDGF was used to study effects on biological activity (1000 units/µg protein) and to compete with labeled PDGF in receptor assays (500 units/µg protein). Human PPP and CM-Sephadex-treated PPP were prepared as described in Ref. 11.

DNA Synthesis. BALB/c-3T3 fibroblasts were plated on micrometer plates (Linbro) in MEM containing FBS (10%), and were allowed to deplete medium for 8 to 10 days. Medium was then removed and changed to assay medium containing PPP (2%), sample, and $[^3H]$thymidine (16 Ci/mmol), carrier-free Na$^{125}$I, $[^3H]$aminosobutyric acid, and [γ-32P]ATP were obtained from New England Nuclear, Boston MA. PDGF was used as previously described (1). PDGF I and PDGF II (3000 units/µg protein) were isolated and separately labeled with $^{125}$I by Iodo-Beads (Pierce Chemical Co., Rockford, IL). Labeled PDGF II was used in binding experiments. Partially purified PDGF was used to study effects on biological activity (1000 units/µg protein) and to compete with labeled PDGF in receptor assays (500 units/µg protein). Human PPP and CM-Sephadex-treated PPP were prepared as described in Ref. 11.

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serum was added and allowed to incubate overnight at 4°C. Biological activity was tested by stimulation of DNA synthesis in BALB/c-3T3 cells. Serum-free conditioned MEM was concentrated by exhaustive dialysis against 1 M acetic acid, lyophilization, and reconstitution in 0.9% NaCl solution containing 1% HSA. This material was tested for stimulation of DNA synthesis in BALB/c-3T3 cells, and was assayed for competition with 125I-PDGF receptor sites on human fibroblasts, as described below.

Radioiodination of PDGF. PDGF I and PDGF II were isolated as described (1). For radioiodination, 5 to 10 μg of PDGF I or PDGF II were dissolved in 100 μl of 0.1 M sodium phosphate buffer, pH 7.0, in a borosilicate test tube (10 x 75 mm). One to 2 ml of 125I (about 5 μCi in 1 M acetic acid, lyophilization, and reconstitution in 0.9% NaCl solution containing 1% HSA) were added, followed by the addition of a single lodo-Bead (Pierce), a chloramine-T-derivatized polystyrene bead. After 40 min, the radioiodinated PDGF was removed and the test tube with the lodo-Bead was rinsed twice with 200 μl of buffer, and once with 200 μl of 0.02% SDS. The radioiodinated PDGF and washes were dialyzed against 0.05 M Tris-HCl, pH 7.2, which was layered over 2 volumes of 35% sucrose: 4 mM Tris buffer, pH 7.2, was removed and cells were incubated for 6 hr at room temperature, with frequent changes of dialysis fluid. After dialysis, the solution (about 1 ml) was placed in an equal volume of 10% HSA. Aliquots (100 μl) were kept frozen. The radioiodinated PDGF was counted on a Packard Model 6230 gamma scintillation spectrometer.

Binding of 125I-PDGF. Binding assays were performed, following modification of the protocol established by Bowen-Pope and Ross (4). MG-63 cells were plated in FBS (10%) on 24-well plates (Flow Laboratories, McLean, VA), grown to confluence, changed to depletion medium (0.5% FBS), and incubated for 72 hr. Labeled 125I-PDGF or unlabelled PDGF or concentrated conditioned medium was added to cells in cold binding medium (0.5% HSA, 4°C). Incubation was carried out for 3 hr at 4°C, either with the culture plates at rest, or while they were being gently shaken. Binding medium was recovered and cells were rinsed 3 times with DPBS. Triton X-100 was originally used to extract bound PDGF, but this yielded incomplete recovery of cell-bound 125I-PDGF. 125I-PDGF was extracted with 1% SDS instead. Nonspecific binding was less than 10% of total PDGF binding, under these conditions. 125I-PDGF was counted on a Packard Model 6230 gamma scintillation spectrometer.

Membrane Phosphorylation. MG-63 cells were grown to confluence in FBS (10%), changed to depletion medium (0.5% FBS), and left for 48 hr. Cells were collected with harvest medium (0.1% EDTA, 0.2 mM phenylmethylsulfonyl fluoride in EBSS) and then pelleted by centrifugation. All subsequent steps were done at 4°C. Cells were resuspended in homogenization buffer (25 mM sucrose: 4 mM Tris: 0.2 mM phenylmethylsulfonyl fluoride, pH 8.4) and homogenized with a Dounce homogenizer. The homogenate was centrifuged (4,000 × g, 10 min) and the supernatant was collected. The pellet was resuspended, homogenized, and centrifuged again. This supernatant was combined with the first supernatant and then centrifuged (30,000 × g for 60 min). The resulting supernatant was discarded and the pellet was resuspended in 4 mM Tris, pH 7.2, which was layered over 2 volumes of 35% sucrose: 4 mM Tris buffer, pH 7.2, and centrifuged (100,000 × g for 60 min.). Plasma membranes were then collected at the resulting interface. Phosphorylation was carried out following a modification of methods previously described (10). Briefly, 20 μg of membrane protein, 30 μl of cDNA incubation buffer (40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid: 20 mM MgCl2: 0.2 mM MnCl2 (pH 7.4), and PDGF were combined and allowed to incubate for 30 min at 4°C. The kinase reaction was initiated by the addition of 0.6 mM ATP (6 to 10 Ci/mmol), and was continued for 20 min at 4°C. The reaction was stopped by addition of SDS sample buffer (4×) followed by boiling 3 min. Samples were then applied to SDS polyacrylamide gel electrophoresis (10%). In some cases, gels were gently shaken in 1 M NaOH (1 hr, 50°C). All gels were fixed and stained with Coomassie brilliant blue. The gels were then dried, and labeled proteins were detected by autoradiography.

Ammonium Transport. Ammonium transport was measured, following the method described by Owen et al. (17). MG-63 cells were plated in FBS (10%) on 24-well plates and were allowed to reach confluence. Medium was changed to FBS (0.5%) and allowed to deplete for 72 hr. Cells were rinsed and exposed to PDGF in MEM for 3 hr. This medium was removed and cells were incubated for 1 hr with DPBS. Cells were then changed to assay medium (0.5 mM [3H]aminosobutyric acid, 8,000 cpm/nmol (DPBS) and were allowed to incubate for 10 min. Cells were then rinsed, solubilized in SDS (0.1%), and the [3H]aminosobutyric acid was counted.

Colony Growth. Cells (3 x 104) were trypsinized and resuspended in 1 ml of FBS (10%). This suspension was diluted 1:1000 in CM-Sephadex-treated PPP (0.5, 1, and 2%), or in FBS (10%) to give 3.5 x 10^6 cells/60-mm dish. Cells were allowed to grow for 2 weeks, with medium changed after 7 days. Cells were rinsed with saline and were fixed with 0.1% methylene blue in methanol. Colonies were counted manually.

Cell Proliferation. MG-63 cells suspended in FBS (10%) were plated at 60,000 cells/well on 24-well plates, and were allowed to attach for 6 hr. Cells were then rinsed with EBSS and transferred into MEM supplemented with PDGF. Medium was changed after 3 days, and cells were counted on Day 6 as previously described (11).

RESULTS

We previously reported that U-2 OS osteosarcoma cells proliferate in PPP in the absence of PDGF (11). The ability of MG-63 cells to form colonies in PPP was tested (Table 1). Plating efficiency in 2% PPP was equivalent to that in 10% FBS, and decreased appreciably with lower concentrations of PPP. Colony size was proportional to PPP concentration, indicating that MG-63 cells proliferate in low concentrations of PPP. One possible explanation for the altered growth requirement of these cells compared to nontransformed mesenchymal cells, which require both PDGF and PPP for maximal growth (2), is endogenous secretion of mitogens. Serum-free medium conditioned by MG-63 cells was found to be mitogenic (Table 2A). Since the U-2 OS osteosarcoma cell line secretes PDGF-like factors, MG-63-conditioned medium was tested for PDGF-like activity. Exposure to rabbit anti-PDGF antiserum did not reduce mitogenicity of MG-63-conditioned medium, indicating that PDGF is not a major mitogen produced by these cells (Table 2A). Higher concentrations of anti-PDGF antiserum were also unable to reduce the mitogenic effect of MG-63-conditioned medium (data not shown). In contrast, anti-PDGF antiserum reduced the stimulatory effect of PDGF.

DiCorleto and Bowen-Pope (8) reported that anti-PDGF antiserum is not capable of blocking mitogenic activity produced by endothelial cells in culture, even though medium conditioned by
these cells can compete with PDGF for membrane receptor sites. Therefore, MG-63-conditioned medium was tested for ability to compete with $^{125}$I-PDG for receptor sites on human diploid fibroblasts (Table 2B). Conditioned medium was exhaustively dialyzed against 1 M acetic acid, lyophilized and reconstituted with HSA (1%) in EBSS. A 1:20 dilution of this concentrated material stimulated acid-insoluble $[^3H]$thymidine incorporation into 3T3 cells (data not shown). The same material could not block $^{125}$I-PDG binding over the concentration range tested. In contrast, unlabeled PDGF significantly reduced specific binding of labeled PDGF. Thus, MG-63 cells do not secrete PDGF-like factors, as measured by competition with labeled PDGF for receptor sites.

Cells of mesenchymal origin have receptors to PDGF (4, 24). Since MG-63 cells do not produce PDGF-like factors and are of mesenchymal origin, it was expected that they would have specific PDGF-binding sites. This was confirmed in binding experiments with $^{125}$I-PDG. In the experiment described in Chart 1, the specific activity of $^{125}$I-PDG was held constant. Scatchard analysis indicated there were 32,000 PDGF receptors/MG-63 cell, with a dissociation constant of $2.4 \times 10^{-11}$ M. In the experiment described in Chart 2, the concentration of labeled PDGF (1.5 ng/ml) was held constant, and various amounts of unlabeled PDGF (0.05 ng/ml to 0.9 pg/ml) were added. Displacement of labeled PDGF occurred when greater than 1.0 ng of unlabeled PDGF/ml was added. The addition of 0.9 pg of unlabeled PDGF/ml resulted in virtually no binding of $^{125}$I-PDG, indicating that the nonspecific binding of labeled PDGF is low in our system, probably due to the purity of the labeled PDGF. Scatchard analysis of these data gave a receptor number and $K_d$ in close agreement with the experiment described in Chart 1 (not shown).

Among the earliest known events modulated by PDGF is the phosphorylation of membrane proteins. PDGF was able to enhance a dose-dependent phosphorylation of a $M$, 185,000 protein in plasma membrane vesicles prepared from MG-63 cells (Fig. 1, A and B). PDGF induced phosphorylation of a membrane protein in human diploid fibroblasts with a similar molecular weight (Fig. 1, C and D). The phosphoprotein from both cell types was stable in 1 M NaOH (Fig. 1, B and D), suggesting that tyrosine is the amino acid residue phosphorylated (5, 15). However, the phosphorylation of MG-63 membrane proteins did differ significantly from phosphorylation in membrane proteins from human diploid fibroblasts. A prominent alkali-stable phosphoprotein with $M$, of 116,000 was evident in MG-63 membranes in the absence of PDGF (Fig. 1B). In the human diploid fibroblasts, prominent alkali-stable phosphoproteins were present only after the addition of PDGF (Fig. 1D).

In other measures of biological activity, PDGF induced similar responses seen in nontransformed cells (2). A dose-dependent increase in $[^3H]$aminolysobutyric acid uptake was observed, as was incorporation of $[^3H]$thymidine and cell proliferation in response to PDGF (Chart 3).

**DISCUSSION**

In experiments presented here, MG-63 osteosarcoma cells did not produce PDGF-like mitogens, in contrast to previous findings with the U-2 OS osteosarcoma cell line. MG-63 cells produced mitogenic activity that was not neutralized by anti-PDGF antisem, nor was capable of competing with $^{125}$I-PDG. Further-
They found that the U-393 OS cell line had PDGF receptors, while the U-2 OS human osteosarcoma cell line did not.

The ability of PDGF to stimulate biological activity in MG-63 cells was tested. PDGF induces a dose-dependent increase in amino acid transport, DNA synthesis, and proliferation. Binding of PDGF to its receptor elicits the same events associated with PDGF-receptor interaction in nontransformed cells (2). Although PDGF may be capable of inducing biological activity in MG-63 cells in the conditions tested, under appropriate conditions, the need for PDGF might be overcome by the production of other growth factors. This is supported by evidence that MG-63 cells secrete mitogens and grow, although not maximally, in PPP treated with CM-Sephadex to remove residual amounts of PDGF. Diploid fibroblasts do not proliferate in low concentrations of CM-Sephadex-treated PPP (11, 19). It was also noted that the addition of anti-PDGF antiserum to MG-63-conditioned medium resulted in greater mitogenic activity than was found in conditioned medium alone. This is probably due to synergy between factors secreted by MG-63 cells and factors present in rabbit serum.

PDGF has been shown to enhance phosphorylation of tyrosine in membrane proteins (10, 16). Of particular interest is the phosphorylation of a M, 165,000 to 185,000 membrane protein which is thought to be the PDGF receptor. The effect of PDGF on the phosphorylation of MG-63 membranes was tested. A dose-dependent increase in M, 185,000 phosphoprotein occurred in MG-63 membranes, which was similar to the enhanced M, 185,000 phosphoprotein levels observed in membranes prepared from human diploid fibroblasts. In order to distinguish the phosphorylation of tyrosine from serine or threonine, phosphorylated proteins were tested for alkal stability (5, 15). When alkal stability was determined, a striking difference was noted between the diploid fibroblasts and the MG-63 cells. In fibroblast membranes, the only alkal-stable phosphoprotein occurred in the presence of PDGF and had a molecular weight of 185,000. In the MG-63 cell membranes, an alkal-stable M, 116,000 phosphoprotein was observed without the addition of PDGF. This suggests that there is a high level of endogenous tyrosine kinase activity in MG-63 cells. These findings may reflect intrinsic differences between transformed and nontransformed cells. It has been noted that some of the acute transforming retroviruses have oncogene products with membrane-associated tyrosine kinase activity (3). Future investigation will attempt to determine if a retroviral oncogene-related mRNA, capable of coding for a tyrosine kinase, is transcribed in these cells. Since peptide growth factors induce tyrosine phosphorylation (2, 6), it is also possible that MG-63 cells secrete a factor unrelated to PDGF which subsequently binds to a membrane receptor and stimulates tyrosine phosphorylation in the M, 116,000 membrane protein. Still another possibility is that membrane kinase activity in the MG-63 osteosarcoma cells is not closely linked to receptor occupancy, resulting in high levels of M, 116,000 phosphoprotein. Although the possibilities are intriguing, there is no concrete evidence that tyrosine phosphorylation is required for cell growth.

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Fig. 1. PDGF-modulated phosphorylation of MG-63 human osteosarcoma and GM-10 human fibroblast cell membranes. Membranes were prepared and phosphorylated as described in "Materials and Methods." A, phosphorylation of MG-63 membranes exposed to PDGF (0, 1, and 25 ng); B, phosphorylation of MG-63 membranes exposed to PDGF (0 to 50 ng), and subsequent alkali treatment of SDS polyacylamide gel; C, phosphorylation of GM-10 membranes exposed to PDGF (0 and 24 ng/ml); D, phosphorylation of GM-10 membranes exposed to PDGF (0 and 24 ng/ml), and subsequent alkali treatment of SDS polyacylamide gel.

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