Binding of Placental Lactogen and Growth Hormone to Fetal Sheep Fibroblasts

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ABSTRACT. Growth hormone (GH) regulates growth and development in the postnatal period but lacks somatotrophic activity in the fetus. In contrast, the placental hormone placental lactogen (PL) stimulates amino acid transport, DNA synthesis, and somatomedin production in isolated fetal tissues, suggesting that PL may function as a "fetal GH." To elucidate the mechanisms by which PL exerts GH-like effects in fetal tissues, we examined the binding of PL, GH, and prolactin to cultured skin fibroblasts obtained from midgestational fetal lambs. Ovine fetal fibroblasts bound radiolabeled ovine PL (oPL) specifically and with high affinity (EC_{50} 0.20 nM). In competitive displacement assays using 125 I-oPL as the radioligand, the potency of unlabeled oPL was eight to 12 times greater than that of ovine GH and ≈ 1000 times greater than that of ovine prolactin. Covalent cross-linking of 125 I-oPL (22 kD) to ovine fetal fibroblasts revealed a specific hormone-receptor complex with an apparent M_r of 130 000, suggesting that the high affinity oPL binding site has a molecular mass of approximately 108 kD. The specific bindings of radiolabeled ovine GH (0.6% per 250 μ g protein) and ovine prolactin (0.04% per 250 μ g protein) were only $1/15$ and $1/230$ that of radiolabeled oPL (9.1% per 250 μ g protein), and no specific cross-linking of 125 I-ovine GH or 125 I-ovine prolactin to ovine fetal fibroblasts was detected. These findings demonstrate preferential binding of PL by isolated fetal sheep fibroblasts in culture, providing a cellular mechanism whereby PL may exert growth-promoting effects in the fetus. (*Pediatr Res* 32: 200–203, 1992)

Abbreviations

GH, growth hormone
PL, placental lactogen
PRL, prolactin
oPL, ovine placental lactogen
oGH, ovine growth hormone
oPRL, ovine prolactin
BS³, Bis(sulfosuccinimidyl)suberate
EC₅₀, 50% effective concentration

The human fetus at midgestation grows at a rate 12 times greater than that of the child in midpuberty (1); however, the regulation of fetal growth by fetal and placental hormones is poorly understood (2). Although GH plays a major role in the control of postnatal growth, it appears to play little or no role in the control of growth in the fetus (2, 3). Several lines of evidence

suggest that PL, a 22-kD polypeptide hormone produced by the fetal placenta, plays a role in the control of fetal growth. PL circulates in fetal plasma from midgestation to parturition in humans, sheep, cows, and mice (for review see Ref. 4) and binds with high affinity to microsomal fractions of ovine (5–9) and human fetal liver (10), bovine endometrium (11, 12), and human fetal skeletal muscle (10). PL exerts metabolic effects on glycogen metabolism in isolated fetal sheep and rat hepatocytes (6, 13–15) and stimulates amino acid uptake, [3 H]thymidine incorporation, and IGF production in human fetal fibroblasts, myoblasts, and pancreatic islet cells (16–18). In the mid- and late-gestation human fetus, plasma PL concentrations correlate positively with fetal plasma IGF-I concentrations and independently with birth weight (19).

The mechanisms by which PL exerts growth-promoting effects in fetal tissues are unknown. To clarify events critical for PL activity at the cellular level, we characterized the binding of oPL to isolated skin fibroblasts from fetal lambs at midgestation. We then compared the binding of oPL to fetal sheep fibroblasts with that of oGH and oPRL.

MATERIALS AND METHODS

Hormones. Binding studies were performed using purified oPL (20) or recombinant oPL (21), the latter a gift from Genentech Inc. (South San Francisco, CA). There were no differences in the binding of the two oPL preparations to fetal sheep fibroblasts or to sheep liver microsomes. oGH (NIH-GH-15) and oPRL (NIH-P-S14) were obtained from the National Hormone and Pituitary Program, National Institute of Arthritis, Digestive Disease, and Kidney. Each hormone was radiolabeled with 125 I-iodine to a specific activity of 50–90 μ Ci/ μ g using 1,3,4,6-tetrachloro-3,6-diphenyl glycouril (iodo-Gen; Pierce Chemical Co., Rockford, IL) as the catalyst (8). Each preparation of 125 I-oPL and 125 I-oGH bound specifically to maternal sheep liver membranes (at least 10% per mg protein) and pregnant rabbit liver membranes (at least 40% per mg protein) during a 2-h incubation at 22°C. The preparation of 125 I-oPRL bound specifically (8–10% per mg protein) to pregnant rat liver microsomes.

Cell culture. Fibroblast cultures were prepared from skin biopsies obtained from 81-d gestation fetal lambs. One-mm fragments were allowed to attach to 25-cm² flasks for 1 h and then overlaid with minimal essential media (Gibco Laboratories, Grand Island, NY) supplemented with 10% defined FCS (Hyclone Laboratories, Logan, UT), 0.1 μ M dexamethasone, 25 μ U/mL penicillin G, 25 μ g/mL streptomycin, 50 μ g/mL gentamicin, and 1 μ g/mL amphotericin B. Fibroblasts were plated in either 6-, 12-, or 24-well culture plates (Costar, Cambridge, MA, and Falcon, Lincoln Park, NJ) and grown to confluence before use in binding or cross-linking studies. Fibroblasts were used between passages 1 and 9.

oPL binding to fetal skin fibroblasts in culture. Confluent fibroblast monolayers were washed three times with 1 mL of cold binding buffer (25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂,

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Fig. 1. Time and temperature dependence of ^{125}I -oPL binding to fetal sheep fibroblasts. Radiolabeled oPL was incubated with fetal sheep fibroblasts in the presence or absence of unlabeled hGH ($10 \mu\text{g/mL}$) at 4°C ($\blacktriangle$), 10°C ($\triangle$), 37°C ($\blacksquare$), or room temperature ($\square$) for various times. Specific binding was calculated by subtracting the amount of binding that occurred in the presence of excess unlabeled hormone (nonspecific binding) from the total binding and expressed as cpm per $250 \mu\text{g}$ protein. Values represent the mean $\pm$ SEM of duplicate values.

Fig. 2. Saturation of the oPL receptor in sheep fetal fibroblasts. Fetal sheep fibroblasts were incubated with various concentrations of ^{125}I -oPL in the absence or presence of unlabeled hGH ($10 \mu\text{g/mL}$) at 4°C for 20 to 24 h. Specific binding was calculated by subtracting the amount of binding that occurred in the presence of excess unlabeled hormone from the total binding. Values represent the mean of duplicate values.

Fig. 3. Comparative binding of ^{125}I -oPL, ^{125}I -oGH, and ^{125}I -oPRL to fetal sheep fibroblasts. Radiolabeled oPL, oGH, or oPRL ($\approx 100,000$ cpm/well) were incubated with fetal sheep fibroblasts in the absence or presence of excess unlabeled hormone for 20 to 24 h at 4°C . Percentage of specific binding was calculated by subtracting the amount of binding that occurred in the presence of excess unlabeled hormone from the total binding and expressed as a percentage of the total radioactivity added per $250 \mu\text{g}$ protein. Values represent the mean of triplicate values $\pm$ SEM. Similar results were obtained in three separate experiments.

2.5 mM MgCl_2 , 11 mM glucose, 0.1% BSA, pH 7.5). Cells were incubated with either radiolabeled oPL, recombinant oPL, oGH, or oPRL (each $\approx 100,000$ cpm, $\approx 0.1 \text{ nM}$) in the presence or absence of unlabeled hormones for various times and temperatures to determine receptor saturability, affinity, and time and temperature dependence. Binding was terminated by washing the cells three times with cold binding buffer; the cells were then

Fig. 4. Effects of unlabeled oPL, oGH, and oPRL on the binding of ^{125}I -oPL to fetal sheep fibroblasts. Radiolabeled oPL ($\approx 100,000$ cpm/well) was incubated with fetal sheep fibroblasts in the absence or presence of increasing concentrations of either oPL, oGH, or oPRL at 4°C for 20 to 24 h. Percentage of specific binding was calculated by subtracting the amount of binding that occurred in the presence of unlabeled hormone from the total binding and expressed as a percentage of the total radioactivity added. Values represent the mean $\pm$ SEM of triplicate values. Curve fitting was generated by a one-site competitive binding curve equation using INPLOT software. Similar or identical results were noted in five separate experiments.

Fig. 5. Cross-linking of ^{125}I -oPL and ^{125}I -oGH to fetal sheep fibroblasts. Fetal sheep fibroblasts were incubated with ^{125}I -oPL or ^{125}I -oGH ($\approx 1 \times 10^6$ cpm/mL) in the presence (+) or absence (-) of oPL, oGH, or oPRL at 4°C for 20 to 24 h. Cross-linking was performed using 0.5 mM DSS as described in Materials and Methods. Solubilized cell extracts were analyzed on a 7.5% SDS-polyacrylamide gel under reducing conditions. Free radioactivity at the bottom of the gel was cut off. Similar or identical results were noted in at least four separate experiments.

solubilized in 1 N NaOH. Radioactivity in the cell-solubilized fractions was determined by gamma scintillation spectrometry.

Cross-linking of ^{125}I -oPL and ^{125}I -oGH to fetal skin fibroblasts in culture. Confluent monolayers of fetal fibroblasts plated in 35-mm^2 wells were washed three times with 2 mL cold binding buffer and then incubated for 20 to 24 h at 4°C with binding buffer containing ^{125}I -oPL, ^{125}I -oGH, or ^{125}I -oPRL ($\approx 1 \times 10^6$ cpm/well) in the absence or presence of unlabeled oPL (200 ng/mL), oGH ($3 \mu\text{g/mL}$), or oPRL ($3 \mu\text{g/mL}$). At the end of the incubation, cells were washed three times with 2 mL cold binding buffer without BSA and were then incubated for 20 min at 4°C in 1 mL of 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 10 mM MgCl_2 (pH 7.5), containing 0.5 mM DSS or 0.5 mM BS³ at 4°C . The cells were then washed extensively in cold binding buffer without BSA and solubilized in $50 \mu\text{L}$ of electrophoresis sample buffer (100 mM Tris, pH 6.8, 4% SDS,

0.2% bromphenol blue, 20% glycerol, 5% β -mercaptoethanol) (22) overnight at 37°C in a humidified chamber on a rocking platform. The individual samples were then boiled for 3 min and analyzed on 7.5% SDS-polyacrylamide gels (Bio-Rad Laboratories, Richmond, CA) at 12 mA/gel. The gels were dried using a vacuum drying apparatus (Bio-Rad) and subjected to autoradiography using Kodak X-OMAT film and intensifying screens for 4 to 7 d at -70°C. The molecular weights of the radioactive hormone-receptor complexes were estimated by comparison with prestained molecular weight standards (Sigma Chemical Co., St. Louis, MO).

Data analysis. The binding of ^{125}I -oPL to fetal sheep skin fibroblasts was examined in the presence or absence of varying concentrations (0.1 ng to 5 $\mu\text{g}/\text{mL}$) of unlabeled oPL, oGH, and oPRL. The total binding of ^{125}I -oPL was expressed as the percentage of radiolabeled hormone bound to cell monolayers. Specific binding was calculated by subtracting the amount of binding that occurred in the presence of excess (10 $\mu\text{g}/\text{mL}$) unlabeled hormone (nonspecific binding) from the total binding. The relative potencies of oPL, oGH, and oPRL in competing for binding to these sites were estimated by analysis of competitive binding data using LIGAND (23) and INPLOT (GraphPAD Software, San Diego, CA) software. In all experiments, the binding of ^{125}I -oPL, ^{125}I -oGH, ^{125}I -oPRL to fetal sheep fibroblasts was determined in either duplicate or triplicate samples and was expressed relative to the protein content of each sample (24).

RESULTS

Time and temperature dependence of ^{125}I -oPL binding to fetal sheep fibroblasts. The binding of ^{125}I -oPL to fetal sheep fibroblasts was time and temperature dependent (Fig. 1). At 37°C, radiolabeled oPL binding was rapid, reaching maximum levels in 30 min to 1 h and then declining precipitously to baseline by 2 h of incubation. The highest specific binding of ^{125}I -oPL was observed during a 24-h incubation at 4°C. Under these conditions, there was no apparent degradation of ligand as assessed by rebinding of the tracer to fetal sheep fibroblasts or by trichloroacetic acid precipitability. All subsequent experiments were performed at 20–24 h at 4°C.

Saturation of oPL binding site in fetal sheep fibroblasts. oPL binding to fetal sheep fibroblasts was saturable during a 24-h incubation at 4°C (Fig. 2).

Comparative binding of oPL, oGH, and oPRL to fetal sheep fibroblasts. To examine the relative potencies of oPL, oGH, and oPRL in competing for the oPL binding site, fetal sheep fibroblasts were incubated with ^{125}I -oPL ($\approx 100\,000$ cpm/well) in the presence or absence of increasing amounts of unlabeled oPL, oGH, or oPRL (Fig. 3). Unlabeled oPL inhibited the binding of ^{125}I -oPL in a dose-dependent fashion, with half-maximal inhibition achieved at a concentration of 0.20 nM oPL. Although oGH also was able to displace ^{125}I -oPL from fibroblast binding sites, the potency of oGH (EC_{50} 1.6–2.4 nM) was only one eighth to one twelfth that of oPL. Similarly, oPRL inhibited ^{125}I -oPL binding only at high concentrations ($\text{EC}_{50} > 200$ nM). In addition, the specific binding of ^{125}I -oGH (0.6% per 250 μg protein) and ^{125}I -oPRL (0.04% per 250 μg protein) to fetal sheep fibroblasts was only $1/15$ and $1/230$ that of ^{125}I -oPL (9.1% per 250 μg protein) (Fig. 4). These findings indicate that fetal sheep fibroblasts bind oPL with high affinity and oGH and oPRL with lower affinities.

Cross-linking of ^{125}I -oPL and ^{125}I -oGH fetal sheep fibroblasts. To characterize further the oPL and oGH binding sites on fetal sheep fibroblasts, cells were incubated with either ^{125}I -oPL or ^{125}I -oGH (each $\approx 1 \times 10^6$ cpm, ≈ 1.0 nM) for 20 to 24 h at 4°C. Nonspecific wells contained an excess of either unlabeled oPL, oGH, or oPRL (Fig. 5). After washing the cells to remove unbound radioligand, radiolabeled hormones were cross-linked to cellular binding sites using either the membrane-permeable

cross-linking reagent DSS or the membrane-impermeable cross-linking reagent BS³. Cross-linking of ^{125}I -oPL (22 kD) to its binding site produced specific radiolabeled complexes of 130, 85, and 60 kD (lane 1). The 130-kD complex was abolished entirely in the presence of 200 ng/mL unlabeled oPL (lane 2) or 3 $\mu\text{g}/\text{mL}$ oGH (lane 3), whereas oPRL was without effect (lane 4). This finding suggests that the M_r of the high affinity oPL binding site in fetal fibroblasts is approximately 108 000. The intensities of the 85- and 60-kD complexes were reduced ≈ 50 –80% by excess unlabeled oPL or oGH, but not oPRL. Studies using DSS or BS³ produced similar cross-linked complexes, suggesting that the cross-linked proteins are associated with the plasma membrane. Cross-linking of ^{125}I -oPL to fetal fibroblasts produced one additional complex noted at the top of the gel (≥ 180 kD). This band may represent a large hormone-receptor complex, aggregated radioligand, and/or aggregated hormone-receptor complexes. No specific cross-linking of ^{125}I -oGH (lanes 5 and 6) or ^{125}I -oPRL to fetal sheep fibroblasts was detected.

DISCUSSION

Although numerous studies have demonstrated that PL exerts direct metabolic and growth-promoting effects in fetal and maternal tissues during pregnancy (4, 6, 13–18, 25–27), the initial events in PL action at the cellular level are unknown. The studies described in this report are the first to characterize the binding of PL to isolated cells in culture, providing new insight into the mechanisms by which the hormone may regulate fetal growth.

oPL bound to fetal sheep fibroblasts specifically and with high affinity (EC_{50} 0.20 nM). The binding of oPL was time and temperature dependent; at 37°C, binding peaked rapidly and then declined, which suggests ligand internalization and/or degradation. Preliminary studies from our laboratory have demonstrated that the oPL receptor in fetal sheep fibroblasts is internalized rapidly after binding oPL at the cell surface; subsequently, the receptor-ligand complex may be recycled back to the cell surface or degraded (28). The highest specific binding of oPL was noted at 4°C, a temperature at which ligand-receptor processing is minimized (29).

The concentrations of oPL required for half-maximal binding to fetal sheep fibroblasts (0.20 nM) were comparable to the concentrations of oPL in fetal sheep serum (25–100 ng/mL, 1.1–4.4 nM) (8, 30, 31); therefore, fibroblast binding sites in the sheep fetus would likely be occupied by oPL under physiologic conditions. Two observations suggest that these sites bind oPL in preference to oGH and oPRL: first, the specific binding of radiolabeled oPL was 15 and 230 times greater than that of radiolabeled oGH and oPRL, respectively; and second, unlabeled oPL was eight to 12 times more potent than unlabeled oGH and ≈ 1000 times more potent than unlabeled oPRL in competing for ^{125}I -oPL binding. The preferential binding of PL to fetal cells in culture may explain in part the differences in potencies of PL, GH, and PRL in various assays of growth-promoting activity in fetal tissues (6, 25–27).

The identity of the oPL binding site in fetal fibroblasts is unclear. Cross-linking of ^{125}I -oPL to intact fetal sheep fibroblasts yielded hormone-receptor complexes of apparent M_r of 130 000, 85 000, and 60 000. The 130-kD radiolabeled complex was abolished by excess unlabeled oPL, which suggests that the high affinity oPL receptor in fetal fibroblasts has an M_r of $\approx 108\,000$. The intensities of the 85- and 60-kD complexes were reduced markedly, but not abolished, by excess unlabeled oPL or oGH, which suggests the presence of lower affinity or nonspecific hormone binding sites. The M_r of the high affinity oPL binding site in fetal sheep fibroblasts (108 000) differs from the apparent M_r of the high affinity oPL receptor in fetal sheep liver membranes and soluble liver extracts (44 000 $\pm$ 2 000) (32–34). The differences in apparent M_r of the oPL binding sites in fetal sheep liver and fetal sheep fibroblasts might be explained in several

ways: first, oPL receptors in various fetal sheep tissues may be heterogeneous in structure, with 44- and 108-kD variants; second, the oPL receptor in fetal sheep liver may be cleaved by proteolysis during the preparation of liver microsomes and detergent extracts, yielding a truncated 44-kD protein that contains the high affinity oPL binding domain; or third, the 108-kD oPL receptor in fetal sheep fibroblasts may represent a complex composed of a binding subunit (44 kD) and a closely associated integral membrane protein (60–64 kD). In this formulation, oPL (22 kD) may cross-link to the binding domain, forming a 60–65-kD complex, or to the complete membrane receptor, forming a 130-kD complex. Nonspecific binding to the associated membrane protein or subunit may produce the 85-kD complex noted in cross-linking studies.

Because the M_r of the oPL binding site in fetal fibroblasts is similar to the M_r of the GH receptor in various mammalian tissues (32, 35), the fibroblast oPL receptor may be a variant of the oGH receptor. Alternatively, the oGH receptor in fetal skin fibroblasts may bind oPL with higher affinity than it binds oGH. The preferential binding of oPL by isolated fetal sheep fibroblasts provides a cellular mechanism by which PL may exert growth-promoting effects in the ovine fetus.

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