Osteogenic Protein-1 Is Produced by Human Fetal Trophoblasts In Vivo and Regulates the Synthesis of Chorionic Gonadotropin and Progesterone by Trophoblasts In Vitro

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Summary: Osteogenic protein-1 (OP-1/BMP-7), a member of the transforming growth factor (TGF-β) superfamily of proteins, is shown to be expressed at sites of epithelial-mesenchymal tissue interaction during human fetal development. In the present study, we examined the expression of OP-1 in human placentas (5—11 weeks of gestation and full term) using in situ hybridization and immunohistochemistry methods. The results show that OP-1 was expressed in cytotrophoblasts (*Langhans'* layer) of the chorionic villi in early and full term placentas. Employing highly purified cytotrophoblast cultures which fused to form functional syncytiotrophoblasts, we showed that exogenously added recombinant OP-1, in the presence of low serum concentration, reduced the secretion of chorionic gonadotropin and progesterone by 60% and 36%, respectively, compared with control cultures. The results suggest that osteogenic protein-1 is synthesized locally by cytotrophoblasts and may be involved in the regulation of human pregnancy by controlling reproductive hormonal secretion.

Introduction

Transforming growth factor-beta (TGF-β) is a superfamily of signalling proteins expressed temporally and spatially at specific sites of morphogenetic events during development. These proteins have been identified in various developmental systems, in both invertebrates and vertebrates. They include the activins and inhibins, osteogenic bone morphogenetic proteins OP-1 to OP-3 (2, 3), BMP-2 to BMP-6 (4, 5), mesoderm inducing factor from *Xenopus laevis* egg (Vg-1) (6), *Muellerian* inhibiting substance (MIS) (7), mammalian protein related to *Xenopus* Vg-1 (BMP-6) (8), growth/differentiation factors (GDF-1, GDF-3, GDF-9) (9, 10) and cartilage derived morphogenetic proteins (CDMP-1 and CDMP-2 or GDF-5 and 6) (11, 12). The decapentaplegic (DPP) and 60A proteins, which are involved in *Drosophila* pattern formations, are homologues of bone morphogenetic proteins (13, 14).

The proteins of the TGF-β superfamily are produced as a precursor that is three times larger than mature protein; the precursor is then processed at proteolytic cleavage sites conforming to the sequence RXXR (3). The C-terminal mature proteins are composed of disulphide-linked dimers with striking identity in the conserved seven cysteine motifs (15). Despite the similar structural features, the temporal and spatial expression of these proteins suggest a functional specificity among them (16—20). Osteogenic protein-1 (OP-1/BMP-7) induces new bone formation in vivo and plays a significant role in cartilage and bone metabolism (21—26).

The presence of high levels of osteogenic protein-1 message in the kidney (19, 27) suggests that OP-1 may have a role in the development of kidney. In addition, the presence of OP-1 in the uterus (28) and a steep decline of OP-1 expression at the onset of pregnancy suggest that reproductive hormones may influence and regulate osteogenic protein-1 expression locally in uterus (28).

In the present study, we examined the distribution of osteogenic protein-1 in human placentas during pregnancy, and examined the effect of OP-1 on the regulation of hormones associated with pregnancy using human trophoblast cultures. The results show that osteogenic protein-1 is expressed and plays a role in regulation of the hormonal secretion during pregnancy.

Materials and Methods

Human tissues

Whole human embryos (gestational age 5—14 weeks) obtained from legal abortions at the University of Zagreb Medical School

were used in this study. The gestational age of each fetus was estimated on the basis of crown-rump length and pregnancy records of the conceptual age. The material was fixed at 4 °C in paraformaldehyde (40 g/l) in 0.1 mol/l phosphate buffer (pH 7.2), embedded in paraffin and cut serially at 5 μm. The procedure for the human autopsy material was approved and controlled by the Internal Review Board of the Ethical Committee at the School of Medicine, the University of Zagreb, and the Office of Human Subjects Research (OHSR) at the National Institutes of Health, Bethesda, MD. The same collection has already been used for localizing bone morphogenetic protein-3 (BMP-3) and OP-1 in skeleton and abdominal organs during human development (17–19).

Mouse tissue

Postimplantation embryos were obtained from outbred females mated with F1 males. At midday the vaginal plug was considered to be 0.5 days p.c. Tissues were fixed at 4 °C in 4% paraformaldehyde in phosphate-buffered saline, and embedded in paraffin. Serial sections were mounted on silanated glass slides, incubated overnight at 40–45 °C, and stored at 4 °C until use.

Probe construction

For the detection of mouse OP-1, a 679 base pair BstXI-BglI-fragment (pO 319-3) covering amino acids 63–263 of the pro-region and the first 25 amino acids of the N-terminal part of the mature polypeptide was used (27). The long fragment was subcloned into a pBluescript II (SK)$^+$-vector, from which the KpnI-Cla and SpeI-SacI multiple cloning site (MCS) fragments were eliminated to prevent cross-hybridization with ribosomal RNA sequences. Based on the comparison of OP-1 probes with other bone morphogenetic proteins, the identity did not exceed 29% [BMP-2 (27%), BMP-3 (28%), BMP-4 (26%), BMP-5 (24%), BMP-6 (29%)], both before and after alkaline hydrolysis, making cross-reactivity unlikely (GeneWorks 2.2.1, IntelliGenetics™, CA). Single-strained sense and anti-sense RNA probes were labelled with [α-^{35}S]UTP (New England Nuclear, Boston, MA) by in vitro-transcription using either SP6 or T7 polymerase.

In situ hybridization

For in situ hybridization, sections were cut at 5–7 μm thickness, mounted on silanated slides, heated at 45 °C overnight, and kept at 4 °C. They were then deparaffinized, rehydrated, postfixed for 20 min in 4% paraformaldehyde and processed as previously described (17, 19). Briefly, pre-treatment included incubation in 0.2 mol/l HCl (5 min), proteinase K-digestion (20 g/l; 10 min), additional postfixation (5 min), blocking of non-specific sulphur-binding sites with iodoacetamide (0.37 g/400 ml; 925 mg/l) and N-ethylmaleimide (0.25 g/400 ml; 625 mg/l), acetic anhydride treatment (5 g/l in 0.1 mol/l triethanolamine-HCl, pH 8.0; 2 × 10 min), and dehydration using a graded series of ethanol. Sections were hybridized under siliconized coverslips for 16–18 h at 50 °C in a humid chamber in hybridization buffer (50% formamide, 10% dextran sulphate, saline/sodium citrate (0.60 mol/l NaCl, 0.060 mol/l Na-citrate, pH 7), 10 mmol/l dithiothreitol, Denhardts solution (200 mg/l bovine serum albumin, 200 mg/l Ficoll, 200 mg/l polyvinylpyrrolidone); 500 mg/l each of salmon sperm DNA and yeast tRNA, and 0.2–0.4 mg/l ^{35}S-labelled asymmetric RNA probes. After hybridization the sections were washed essentially as described earlier (17), including the following steps: 15 min in saline/sodium citrate (0.30 mol/l NaCl, 0.030 mol/l Na-citrate), 50 °C; 20 min in 50% formamide Na-citrate (0.30 mol/l NaCl, 0.030 mol/l Na-citrate, 20 mmol/l dithiothreitol), 65 °C; 2 × 10 min in Tris/EDTA/NaCl buffer (10 mmol/l Tris-HCl, pH 7.5; 5 mmol/l EDTA, 0.5 mol/l NaCl), 37 °C; 30 min in Tris/EDTA/NaCl buffer containing 20 mg/l RNase A, 37 °C; 10 min in Tris/EDTA/NaCl buffer, 37 °C; 2 × 15 min in saline/sodium citrate (0.30 mol/l NaCl, 0.030 mol/l Na-citrate), 65 °C; and finally to 15 min washes in saline/sodium (0.15 mol/l NaCl, 0.015 mol/l Na-citrate), 65 °C. Sections were then dehydrated through an upgraded series of ethanol

containing 0.3 mol/l ammonium acetate, covered with NTB-2 emulsion (Kodak), and exposed between 1 and 3 weeks. After development, the slides were stained with 1 g/l toluidine blue, dehydrated, cleaned with xylene and mounted with Permount.

OP-1 antibodies production

Several polyclonal and monoclonal antibodies that recognize specifically the mature osteogenic protein-1 molecule have been produced and characterized (19). Briefly, the polyclonal rLOP antibody was generated in rabbits using the mature domain of osteogenic protein-1 subunit (amino acids 293–431) (2) expressed in Escherichia coli as immunogen. The polyclonal SER-17 antibody was generated in rabbits using a synthetic peptide of 17 amino acids representing the N-terminus of mature osteogenic protein-1 monomer (amino acids 293–309) (2). Two monoclonal antibodies, 1B12-D3 and 12G3, capable of neutralizing osteogenic protein-1 activity were also used for immunohistochemical staining.

Preparation of dispersed mononuclear trophoblasts

Normal placentas (8–12 weeks gestation) were obtained immediately after therapeutic procedures and processed according to the modified method of Kliman et al. (29). The tissue was rinsed in sodium chloride (9 g/l) containing bacitracin (Serva, Heidelberg, Germany; 4 mg/l) and leupeptin (Boehringer, Mannheim, Germany; 3 mg/l). The specimens were then dissected free of connective tissue and blood vessels, coarsely minced and transferred to 20 ml pre-warmed calcium-magnesium free Hanks' solution (CMF Hanks'; Sigma Diagnostics, St. Louis, USA), containing 25 mmol/l HEPES (Sigma), 0.75 g/l trypsin (Sigma; 1070 U/mg) and 0.6 g/l DNase I (Sigma; 530 Kunitz units/mg), pH 7.4, and finally incubated in a shaking water bath at 37 °C for 20 min. Tissue fragments were allowed to settle for 1 min. Supernatant (15 ml) was removed, recovered in medium containing fetal calf serum (Gibco, Gaithersburg, USA) and centrifuged at 300 g for 5 min at room temperature. The resultant pellet was resuspended in 5 ml α-MEM (Sigma) containing 25 mmol/l HEPES, fetal calf serum, volume fraction 0.1, and 25 mmol/l glucose and stored at 4 °C. The remaining placental tissue was digested four more times with the addition of 20 ml pre-warmed trypsin-DNase solution. Five resultant cell suspensions were pooled, centrifuged at 300 g for 5 min, and resuspended in 3 ml α-MEM-HEPES-glucose.

Culture of trophoblasts

The suspension of isolated cells was layered over a preformed Percoll gradient made up in Hanks' solution. The gradient consisted of 70% to 20% Percoll (vol/vol) in 10% steps of 3 ml each, by dilutions of 90% Percoll [9 parts Percoll (Pharmacia, Uppsala, Sweden): 1 part 10 times concentrated Hanks'] with calcium-magnesium free Hanks' and layered in a 25 ml conical polystyrene centrifuge tube. The gradient was then centrifuged at 400 g at room temperature for 20 min and three regions were identified, namely:

(1) red blood cells and occasional polymorphonuclear leukocytes at the bottom,

(2) connective tisssue elements, small vessels and villous fragments at the top, and

(3) mononuclear cells in the middle.

The middle layer was removed, washed once with α-MEM-HEPES-glucose, resuspended and diluted to a concentration of 3 × 10^8 cells per litre with α-MEM containing glutamine (Flow, Bucks, England; 292.3 g/l), penicillin and streptomycin (Sigma; 100 000 U/l, 0.1 g/l), and fetal calf serum, volume fraction 0.15. The cells were plated into 96-well plates (Flow; 200 ml, 60 000 cells/well) incubated in a humidified atmosphere with 5% CO_2– 95% air at 37 °C for 24 hours. The culture medium was then supplemented with 1 g/l bovine serum albumin and 1% ITS + TM premix [Collaborative Research Inc., Bedford, USA; containing insulin (6.25 g/l), transferrin (6.25 g/l), selenious acid (6.25 g/l), bovine serum albumin (1.25 g/l), linoleic acid (5.35 g/l)], osteogenic

Fig. 1 OP-1 expression in the murine pregnant uterus by in situ hybridization.
Hybridization was done with a 679 base pair *BstXI-BglI* OP-1 specific riboprobe.

(A) Transverse toluidine-blue stained brightfield section of a 7-day pregnant uterus. Arrows indicate layers of giant cytotrophoblast adjacent to maternal placenta. The developing embryo is in the middle of the section. Magnification: × 60
(B) Darkfield image of an adjacent section with transcripts localized in the cytotrophoblasts of the placenta (arrows). Magnification: × 60

(C) Toluidine-blue stained section of uterus containing the fetus of a 10-day pregnant mouse. Arrows indicate trophoblastic giant cells. Magnification: × 60
(D) An adjacent darkfield section with a strong signal in the trophoblastic giant cells (arrows). Bar in (d) indicates 100 μm in (a—d). Magnification: × 60

protein-1 [Creative BioMolecules, Hopkinton, MA, USA (20 mg/l)] and bone morphogenetic protein-2 [Creative BioMolecules, Hopkinton, MA, USA, (20 mg/l)]. At designed times, culture media were removed, frozen at −70 °C and replaced with fresh medium. Cells were cultured for up to 5 days. Experiments following each isolation of trophoblasts were done in triplicate, with 12 wells per group.

Immunostaining methods

Sections were deparaffinized and then treated with 1000 U/l chondroitinase ABC (Sigma Diagnostics, St. Louis, USA) for 10 min at 37 °C to facilitate the antibody penetration into the tissues. This was followed by exposing the sections to 1 : 30 dilution of normal goat serum (Amersham, Buckinghamshire, England) for 30 min. Sections were then incubated with a dilution of the primary antibody in TRIS-buffered saline (15 mg/l protein), 5 g/l bovine serum albumin (Sigma), overnight at 4 °C. After washing in TRIS-buffered saline containing 1 g/l bovine serum albumin, sections were incubated for one hour with an affinity-purified secondary antibody, linked to colloidal gold particles of about 5 nm mean diameter (Auroprobe LM; Janssen, Olsen, Belgium). For control experiments, the primary antibody was replaced with either

(a) bovine serum albumin,
(b) normal rabbit serum,
(c) IgG preabsorbed with osteogenic protein-1,
(d) secondary antibody alone or
(e) by using the antigen-depleted IgG preparations.

Hormone quantification

For the determination of hormone production by cultured trophoblasts conditioned media were collected and stored at −70 °C until analysed. Oestradiol was determined by a radioimmunoassay, progesterone was measured using an enzyme immunoassay, and the β-subunit of human chorionic gonadotropin by immunoenzymetric assay with magnetic solid phase (Serono diagnostics, Coinsins, Switzerland).

Statistics

All results are expressed as mean ± SEM. Data (12 wells per experimental group) were tested by *Student*'s t-test, followed by two-

way analysis of variance (ANOVA). Means were then tested by the *Scheffe*-post-hoc test. *Student*'s t-test, ANOVA and *Scheffe*-post-hoc tests were performed with the "Crunch" statistical package.

Fig. 2 Specificity of osteogenic protein-1 staining.

(A) Parasagittal section through the placenta of a 22 mm embryo. The *rLOP* antibody to osteogenic protein-1 causes intense staining of the chorionic cell layer (arrows). Magnification: × 25
(B) An equivalent aliquot of antibody preincubated with a solution to which osteogenic protein-1 had been added gave no staining (arrows). The dilution of the anti-osteogenic protein-1 IgG preparation was 1 : 150. Immunogold staining with light green counterstain. Bar indicates 20 μm. Magnification: × 25

Results

Osteogenic protein-1 expression in murine trophoblasts

At 7 to 10 days of murine pregnancy, the OP-1 mRNA expression was observed mainly in trophoblastic giant cells of the fetal placenta (fig. 1a–d) and remained in cytotrophoblasts during subsequent development (data not shown). There were no detectable OP-1 transcripts in endometrium constituting the maternal placenta. Localization of OP-1 in fetal tissues is in accordance with our previous observations (17, 19).

Localization of osteogenic protein-1 in human placenta during pregnancy

Specificity of immunostaining was initially tested by using the polyclonal rLOP antibody raised against mature OP-1 monomer and then by employing the polyclonal SER-17 (raised against N-terminal peptide of mature OP-1) and monoclonal 1B12-D3 antibodies (raised against mature OP-1 dimer) (18). The specificity of immunostaining to osteogenic protein-1, was demonstrated by preincubating the anti-osteogenic protein-1 IgG fractions with a solution of 5 mg/l of recombinant human osteogenic protein-1 before use. This procedure resulted in a complete loss of staining, thus demonstrating that the osteogenic protein-1 immunoreactivity is specific (fig. 2a, b). Since the placenta continuously undergoes changes in size, shape and internal structure during gestation, osteogenic protein-1 distribution was examined in placentas from 2, 3, 6, 13, 27 and 33 mm

embryos, as well as in full term placentas. Intensive cytoplasmic staining was observed in the chorionic cell layers by the polyclonal rLOP antibody (fig. 3a, b). Osteogenic protein-1 was detected in the cytotrophoblast layer (Langhans' layer) (fig. 3d) of chorionic villi, which reach the peak of their development during the second month and regress from the 13th week onwards. Hofbauer cells (fig. 3d), which appear to be phagocytic and are commonly believed to act as a primitive type of macrophage, also stained for osteogenic protein-1. In full term placentas, the remaining cytotrophoblasts still stained for osteogenic protein-1. These findings suggest a role for osteogenic protein-1 in early enbryogenesis and remodelling of the placenta.

Osteogenic protein-1 inhibits hormonal secretion of cultured human trophoblasts

Highly purified functional cytotrophoblasts were prepared from early and full-term human placentas. The lack of immunogold staining with antibodies against vimentin, von Willebrand-factor VIII and α_1-antichymotrypsin confirmed Kliman's data (29) that endothelial cells, fibroblasts, or macrophages did not contaminate the purified cytotrophoblast preparations. Figure 4 shows isolated human cytotrophoblasts in culture and formation of human chorionic gonadotropin-positive syncytia over a period of 18–66 hours. Hormonal secretion was detected 24 hours after culturing the cells, which correlated with the beginning of cell aggregation. Isolated human trophoblasts showed a steadily increasing synthesis of human chorionic gonadotropin and pro-
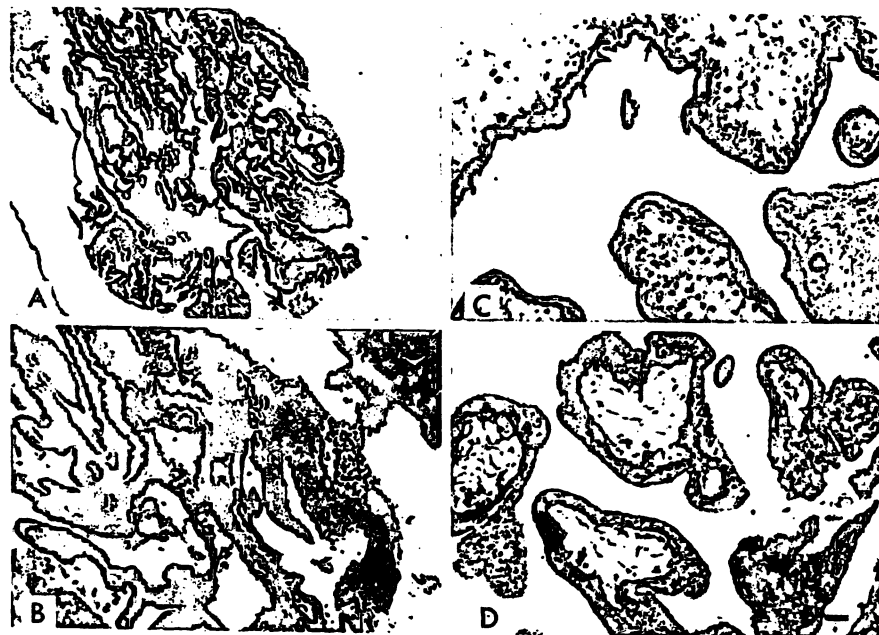

Fig. 3   Localization of osteogenic protein-1 in early human placenta.

(A) A chorionic cell layer of a 27 mm embryo stains intensely for osteogenic protein-1 with rLOP antibody. Magnification: × 5
(B) Higher magnification of (a). Magnification: × 25
(C) Chorionic villi from the placenta of a 13 mm embryo. Osteogenic protein-1 is detectable in the cytotrophoblast cell layer (arrows.). Magnification: × 25

(D) Staining of full term placental tissues with a monoclonal 1B12-D3 antibody. Remaining cytotrophoblasts continue to show the presence of osteogenic protein-1 (arrows). Immunogold staining with light green counterstain. Magnification: × 25.
Bar indicates 100 μm in (A), 20 μm in (B, C, D).

gesterone, reaching maximal values by 96 hours in the presence of fetal calf serum, volume fraction 0.15 (fig. 5a, b). To test the possible effect of osteogenic protein-1 on hormonal activity of isolated trophoblast cells, different amounts (1−100 mg/l) of osteogenic protein-1 were added to cultured cells 24 hours after plating. Both

chorionic gonadotropin (fig. 5a) and progesterone (fig. 5b) secretion into the conditioned media were decreased by 60% and 36% at 72 hours, respectively. The inhibition was maximal in the presence of 20 mg/l osteogenic protein-1 and most effective in the presence of 5 ml/l of fetal calf serum. At a high serum content (150 ml/l) in
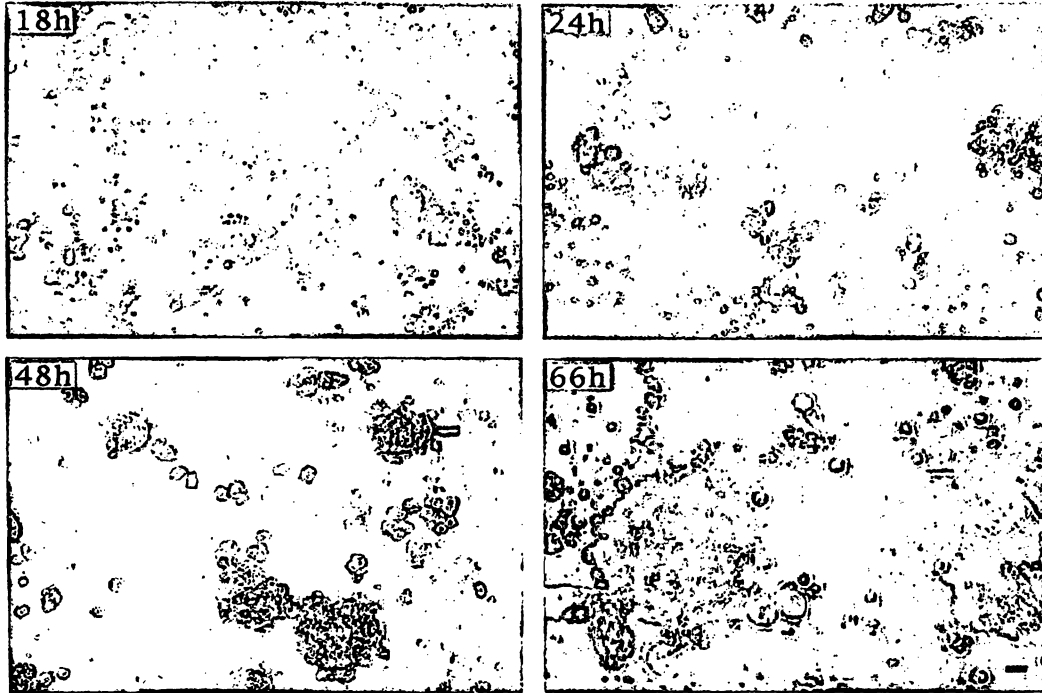

Fig. 4 Human trophoblasts in culture.
Highly purified functional cytotrophoblasts were isolated from early human placentas and grown in α-MEM containing glutamine and 150 ml/l fetal calf serum for up to 5 days as described in Material and Methods. Cells were seeded at 300 x10⁶/l in 96-well

plates (60 000/well). Cell aggregates formed as early as 24 hours after plating; these eventually fused to form functional syncytiotrophoblasts 24−48 hours after plating. Bar indicates 500 μm in (18 h), 380 μm in (24 h), 250 μm in (48 h) and 100 μm in (66 h). Magnification: X 12 (18 h), X 32 (24 h, 48 h), X 50 (66 h)
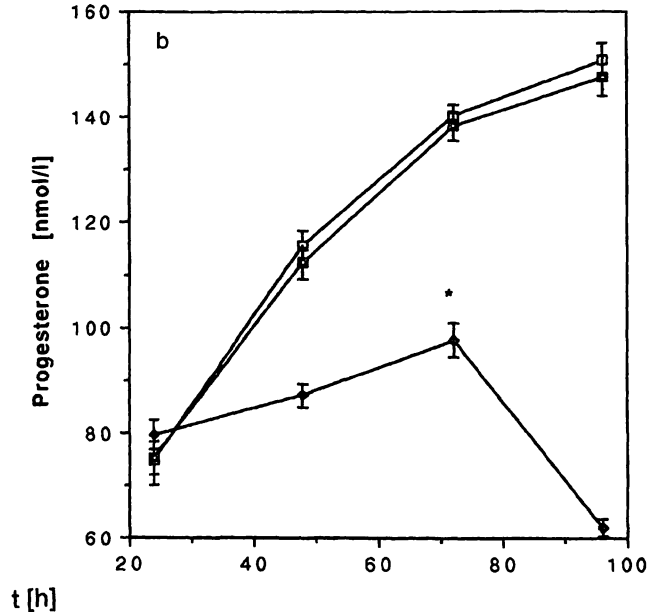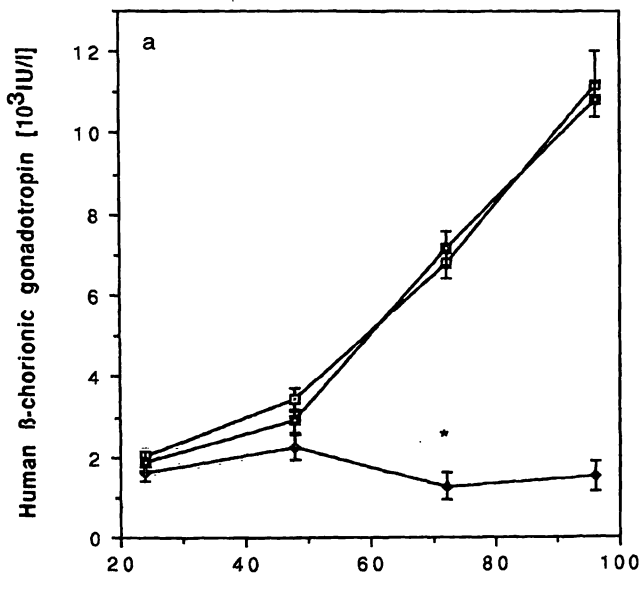

Fig. 5 Hormonal secretion by human trophoblasts treated with a single dose of osteogenic protein-1. Cells were cultured in α-MEM containing glutamine and 5 ml/l fetal calf serum for up to five days in 96-well plate (60 000/well), with the addition of osteogenic protein-1 or bone morphogenetic protein-2 as indicated. Addition of a single dose of 20 mg/l osteogenic protein-1 24 hours after plating the cells reduced the secretion of

(a) chorionic gonadotropin in trophoblast conditioned media by 60%,
(b) progesterone secretion by 36%.
Bone morphogenetic protein-2 (recombinant human BMP-2, 20 mg/l) had no effect on hormone secretion. Experiments were done in triplicate, with 12 wells per group (n = 12).
* P < 0.01 vs control.

—□— control   —♦— rhOP-1   —■— rhBMP-2

the presence of various amounts of osteogenic protein-1, the differences were less pronounced (data not shown). The most effective reduction in the secretion of chorionic gonadotropin at low fetal calf serum concentration was achieved by the addition of 20 mg/l of osteogenic protein-1 immediately after plating (fig. 6). Low chorionic gonadotropin values were maintained throughout the experiment by the daily addition of osteogenic protein-1.

Discussion

The present study identifies cytotrophoblasts in the human fetal placenta as a site of OP-1 synthesis during pregnancy. Moreover, OP-1 seems to be involved in the regulation of secretion of chorionic gonadotropin and
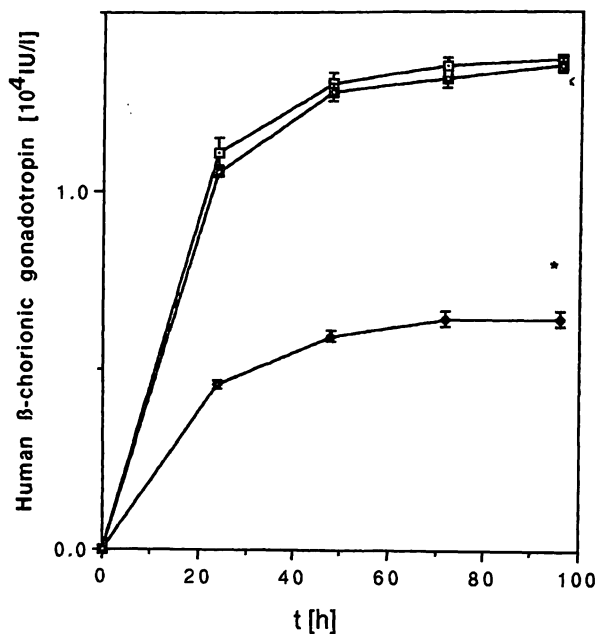

Fig. 6 Hormonal secretion by human trophoblasts treated daily with osteogenic protein-1. Cells were cultured in α-MEM containing glutamine and 5 ml/l fetal calf serum for up to five days in a 96-well plate (60 000/well), with the addition of osteogenic protein-1 or bone morphogenetic protein-2 as indicated. Administration of osteogenic protein-1 (20 mg/l) immediately after plating, caused a pronounced decrease in chorionic gonadotropin secretion by human trophoblasts within 24 hours. In parallel, bone morphogenetic protein-2 (recombinant human BMP-2, 20 mg/l) had no effect on hormone secretion. Experiments following each isolation of trophoblasts were done in triplicate, with 12 wells per group (n = 12).
* P < 0.01 vs control.
−□− control   −◆− rhOP-1   −■− rhBMP-2

progesterone by cytotrophoblasts. During pregnancy both placental cytotrophoblasts and the developing embryo synthesize OP-1. Expression of OP-1 appears to correlate with the biochemical changes associated with trophoblasts, as evidenced by the secretion of increased amounts of chorionic gonadotropin and its peak values during the first trimester of pregnancy. The specific activity of OP-1 on placental hormone secretion was further corroborated by the failure of morphogenetic protein-2 to inhibit the same assay system. Analysis of the spatial and temporal expression of OP-1 patterns during development showed a widespread distribution of OP-1 expression at the sites of specific developmental events involving epithelial mesenchymal interaction (20). The detection of OP-1 message in tissues like the kidney, heart, early limb buds, whisker follicles and teeth, indicates that OP-1 may have, apart from bone induction, important signalling or regulatory roles during epithelial-mesenchymal interactions. This has been previously suggested for other members of the TGF-β superfamily (17). Although both OP-1 and BMP-2 induce new bone and cartilage formation at ectopic sites and share type I and II, two cell surface receptors, their specific functional roles during development are yet to be discovered. This system provides a good basis for delineating the apparent functional differences among the bone morphogenetic protein family members.

Since pregnancy and/or the administration of oestrogen regulates the expression of osteogenic protein-1 in the uterus, cytotrophoblasts and fetal organs, it is possible that the osteogenic protein-1 gene may have tissue-specific regulatory elements in the upstream 5' characterization of promotor region. Upstream regulatory sequences in the osteogenic protein-1 genomic locus, with specific binding sequences for transcriptional regulatory protein would provide a better understanding of the temporal and spatial regulation of OP-1 during tissue morphogenesis. Recent studies on deletion of the OP-1 gene suggest that mice carrying the null mutation died due to lack of a functional kidney, as well as displaying several skeletal abnormalities and dysfunction of the fetal placenta, resulting in a small, underdeveloped animal (Karsenty et al., in preparation). These observations further support our conclusion that OP-1 has a regulatory role in the placental cytotrophoblasts during pregnancy.

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