Amniotic fluid and plasma levels of parathyroid hormone-related protein and hormonal modulation of its secretion by amniotic fluid cells

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Parathyroid hormone-related (PTHrP), the major mediator of humoral hypercalcemia of malignancy, may also regulate placental calcium flux, uterine contraction and fetal tissue development. In the present study, we demonstrated that the mean immunoreactive PTHrP concentrations in amniotic fluid at mid-gestation (21.2 ± 3.7 pmol/l) and at term (19.0 ± 2.7 pmol/l) were 13–16-fold higher than levels measured in either fetal (1.6 ± 0.1 pmol/l) or maternal plasma (1.4 ± 0.3 pmol/l) at term and equal to levels found in plasma of patients with humoral hypercalcemia of malignancy. In vitro studies pointed to three possible sources of PTHrP in amniotic fluid: cultured amniotic fluid cells, cells derived from the amniotic membrane overlying the placenta and placental villous core mesenchymal cells. Treatment of cultured amniotic fluid cells with human prolactin, human placental lactogen (hPL) or human growth hormone (100 µg/l) increased PTHrP secretion after 24 h by 43%, 109% and 90%, respectively. Insulin-like growth factors I and II (100 µg/l), insulin (100 µg/l) and epidermal growth factor (EGF) (10 µg/l) increased PTHrP secretion by 53%, 46%, 68% and 118%, respectively. The stimulation of PTHrP secretion by EGF or by hPL was both time- and dose-dependent. In contrast, calcitriol and dexamethasone (10 nmol/l) decreased PTHrP secretion by 32% and 75%, respectively. Estradiol, progesterone, dihydrotestosterone and human chorionic gonadotrophin had no effect on PTHrP secretion. These findings support the notion that PTHrP may play a physiological role in the uteroplacental unit and demonstrate that human amniotic fluid cells could be a useful model for studying the regulation of PTHrP production and secretion by hormones and growth factors.

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Parathyroid hormone-related protein (PTHrP) is the primary endocrine factor responsible for the syndrome of humoral hypercalcemia of malignancy (1, 2). Both PTHrP and PTHrP mRNA have also been localized in a wide range of normal tissues, such as skin (3), lactating mammary gland (4), uterus in pregnancy (5) and pancreas (6). Although the physiological role of PTHrP has yet to be defined, it has been suggested that the protein may act predominantly as a paracrine/autocrine factor affecting differentiation of cells and also as a smooth-muscle relaxant (7–10).

Recent studies indicated that PTHrP may also regulate fetal calcium homeostasis, uterine contraction and fetal tissue development (8, 11, 12). PTHrP, a potent inhibitor of uterine contraction (12), is produced in the pregnant rat myometrium (5) and in the human amnion (13, 14) and accumulates in human amniotic fluid (14). Production is dependent upon intrauterine occupancy and decreases following rupture of the membranes and onset of labor (5, 14). Other studies indicated that PTHrP has a physiological role in regulating the transport of calcium from the maternal to the fetal circulation (11, 15).

In this report, we have extended these studies by comparing amniotic fluid levels of immunoreactive PTHrP and intact PTH with circulating levels of these hormones in the mother and in the fetal umbilical artery and vein. In addition, the present studies suggest three possible sources of PTHrP in the amniotic fluid—amniotic fluid cells, cells from the amnion tissue and placental villi core mesenchymal cells—and demonstrate the regulation of PTHrP production in cultured amniotic fluid cells by a variety of hormones and growth factors.

Subjects and methods

Sample collection

Amniotic fluid samples were obtained from 15 women...
in their 17th week of gestation undergoing amniocentesis for detection of chromosomal aberrations. Twenty amniotic fluid samples were also obtained at term during cesarean sections or vaginal deliveries. In addition, fluids from first-trimester amniotic sacs were obtained from four women during selective reduction of multiple pregnancy at 10–11 weeks of gestation. Plasma samples were collected from 10 healthy young women from our hospital staff, from 12 of the pregnant women at mid-gestation, from nine of the pregnant women at term and from 10 newborn babies (15.2 ± 3.8 h old). Plasma samples were also obtained from the umbilical cord veins and arteries of 12 newborn babies at the time of delivery. Amniotic fluid samples (2 ml) and 2-ml blood samples were drawn directly into collecting tubes containing protease inhibitors (Nichols Institute Diagnostics, San Juan Capistrano, CA), centrifuged immediately at 4°C and stored frozen until assayed. The study was approved by the Human Investigation Committee of the Tel-Aviv Medical Center.

Cell and tissue cultures

Amniotic fluid samples (10–20 ml) were centrifuged at 800 g for 10 min to obtain cells that were cultured in Medium-199 (Biological Industries, Kibbutz Beth Haemek, Israel) supplemented with 20% fetal calf serum (FCS), glutamine (2 mmol/l), penicillin (100 µ/ml), streptomycin (200 mg/l) and mycostatin (12 µ/ml). After establishment of cultures, cells not used for genetic studies were subcultured in Medium-199 supplemented with 10% FCS in humidified air with 5% CO₂. Amniotic fluid cells are heterogeneous mixture of cells (fetal skin, fetal lung, fetal urinary tract and amnion) that are the most readily accessible sources of fetal human cells. Villous core mesenchymal cell cultures were established after enzymatic dissociation of first-trimester placental villi. Placental samples were obtained from women undergoing therapeutic abortions by dilatation and curettage at 9–11 weeks of pregnancy. Placental villi were washed free of blood in RPMI-1640 medium (Biological Industries, Beth Haemek, Israel) and incubated for 1 h with 0.25% trypsin−EDTA (Biological Industries, Beth Haemek, Israel) at 37°C. The villi were washed and dissociated further for 20 min in RPMI-1640 medium containing 120 U/ml collagenase (type 1S, Sigma Chemical Co.) at 37°C. The cells were washed, pelleted, resuspended and cultured in Chang tissue culture medium (Irvine Scientific, Santa Ana, CA). After 48 h, the non-adherent cells in the medium (mainly trophoblasts) were removed and incubated under non-adherent conditions in RPMI-1640 medium for an additional 48 h period and then centrifuged and pelleted. The supernatant contained high levels of hCG (> 500 µU/ml) and human placental lactogen (hPL > 1.0 mg/l), confirming the identity of these cells as trophoblasts. Samples of the supernatants were frozen at −20°C until assayed for PTHrP. In contrast, the adherent mesenchymal-like cells produced neither hCG nor hPL. Cultured amnion tissue cells were obtained from amnion overlaying the placenta and from reflected amnion obtained at term and prepared as described elsewhere (16). All cell cultures were used when they were 90% confluent and had been incubated for an additional 24 h in medium containing 2% FCS, after which the culture media were collected for immunoradiometric assay of PTHrP. Tissue samples of term placenta, decidua and amnion were cut into small pieces (2 mm in all dimensions; 80 mg/dish) and incubated in RPMI-1640 medium with 2% FCS for 24 h. Then, the media were collected and assayed for PTHrP.

To examine the effects of hormones and growth factors on PTHrP production, the media containing 10% FCS were changed to media containing only 2% FCS, and cultures of amniotic fluid cells and of placental mesenchymal cells were treated for 24 h with one of the following hormones or growth factors: dexamethasone (10 nmol/l; Teva Pharmaceutiels Industries, Jerusalem, Israel), calcitriol (10 nmol/l; donated by Hoffmann-LaRoche Co., Basel, Switzerland), estradiol (30 nmol/l), dihydrotosterone (300 nmol/l), progesterone (1 µmol/l) and epidermal growth factor (EGF, 10 µg/l) (Sigma Chemical Co., St Louis, MO), hPL and hPRL (100 µg/l; National Hormone and Pituitary Distribution Program NIDDKD, NH), rhGH (100 µg/l; Biotechnology-General, Nes-Ziona, Israel), hCG (100 µg/ml, Teva Pharmaceuticals, Israel), IGF-I and IGF-II (100 µg/l; donated by CIBA-Geigy, Basel, Switzerland) and insulin (100 µg/l; Novo Industries, Denmark). In separate experiments we measured the time course and dose-dependent effect of EGF and hPL on PTHrP secretion by amniotic fluid cells.

The media were collected and stored at −20°C until assayed for PTHrP. Cell counts were performed in all experiments after the media were collected and the PTHrP concentration was expressed per 10⁵ cells.

Assays for PTHrP and intact PTH

Concentrations of PTHrP in amniotic fluid, plasma and culture media were measured by a two-site immunoradiometric assay (IRMA) for the measurement of the 1–86 amino acid sequence of PTHrP (Allegro-PTHrP immunoassay kit, Nichols Institute Diagnostics, San Juan Capistrano, CA). This assay is specific for human PTHrP and does not cross-react with various human PTH fragments. The intra-assay coefficient of variation was 9% and the interassay variance was 12%. Intact PTH was measured by a two-site IRMA (Allegro intact PTH kit, Nichols Institute Diagnostics, San Juan Capistrano, CA). The interassay and intra-assay coefficients of variation were 9% and 6%, respectively.
Statistics
Results are expressed as means ± SEM. Comparisons were done using Student's unpaired t-test.

Results
Concentrations of PTHrP and intact PTH in amniotic fluid and plasma
The concentrations of immunoreactive PTHrP in amniotic fluid at the 17th week of gestation (21.1 ± 3.7 pmol/l) and at term (19.0 ± 2.7 pmol/l; fig. 1) were 13–16-fold higher than those measured in plasma of the pregnant women (1.6 ± 0.1 pmol/l at mid-gestation and 1.4 ± 0.3 pmol/l at term: fig. 1) or those in the young control women (1.3 ± 0.2 pmol/l: Fig. 2). These concentrations of amniotic fluid PTHrP were comparable to those found in plasma of patients with humoral hypercalcemia of malignancy. No differences were found at term between amniotic fluid PTHrP levels of labored vs non-laboring women or between vaginal samples and those obtained at cesarean sections. The concentration of PTHrP was already elevated significantly (9.1 pmol/l) in one sample obtained at the 11th week of gestation (Fig. 1), no correlation was found between amniotic fluid and maternal plasma PTHrP concentrations. The mean PTHrP concentration in umbilical cord venous plasma (1.6 ± 0.1 pmol/l) did not differ from values determined in plasma from the umbilical cord artery (1.6 ± 0.1 pmol/l: Fig. 2). The mean plasma PTHrP concentration in newborn babies was 0.7 ± 0.2 pmol/l (Fig. 2).

In contrast to PTHrP, the mean concentrations of immunoreactive intact PTH in amniotic fluid at mid-gestation (11.7 ± 1.5 ng/l) and at term (5.5 ± 1.0 ng/l) were lower than those found in maternal plasma at mid-gestation (15.8 ± 3.4 ng/l) and at term (18.7 ± 3.1 ng/l), and both were lower or at the lower range of normal values in normal human serum (range 15–65 ng/l). Immunoreactive intact PTH levels were suppressed in both venous (1.4 ± 0.2 ng/l) and arterial (1.4 ± 0.1 ng/l) umbilical cord plasma samples.

Secretion of PTHrP by cell and tissue cultures
To determine the source(s) of amniotic fluid PTHrP, we investigated the in vitro secretion of PTHrP by cell and tissue cultures prepared from the placenta and associated membranes and by cultured amniotic fluid cells. We identified three possible sources for PTHrP in amniotic fluid: cultured amniotic fluid cells (16.8 ± 0.2 pmol·I⁻¹·10⁻⁵ cells), placental villi core mesenchymal cells prepared from placental villi samples obtained at the 10th week of gestation (23.9 ± 0.4 pmol·I⁻¹·10⁻⁵ cells) and cells prepared from the amniotic membrane overlying the placenta (10.5 ± 0.6 pmol·I⁻¹·10⁻⁵ cells). No immunoreactive PTHrP was detected in medium from cultured non-adherent cytotrophoblasts or in media of explants of term placenta, decidua and amnion.

Treatment of cultured amniotic fluid cells with hPRL, hPL and hGH (100 μg/l) for 24 h increased the PTHrP concentration in the culture media by 43%, 109% and 90%, respectively (Fig. 3a). Insulin-like growth factor I (IGF-I) and IGF-II (100 μg/l), insulin (100 μg/l) and EGF (10 μg/l) increased PTHrP secretion by 46%, 68%, 53% and 118%, respectively (Fig. 3b). In contrast, calcitriol and dexamethasone (10 nmol/l) decreased PTHrP concentration in the culture media by 32% and 75%, respectively (Fig. 3c). Estradiol, dihydrotestosterone, progesterone and hCG had no effect on PTHrP secretion. The stimulation of PTHrP secretion by EGF or hPL was both time- and dose-dependent (Figs. 4 and 5). A significant increase in PTHrP secretion occurred as early as 2 h after stimulation by EGF or hPL, and a maximal increase was sustained between 8 and 24 h. A significant response was reached at both 1 μg/l EGF and 10 μg/l hPL. Cycloheximide (10 ng/l) inhibited the increase in PTHrP concentration in the culture media following EGF or hPL stimulation (data not shown), suggesting that the effect of EGF and hPL was due to de novo protein synthesis. Human PL, hPRL, hGH, EGF and dexamethasone did not affect PTHrP secretion by placental villous mesenchymal cells.

Discussion
In the present study, we demonstrated that the concentrations of immunoreactive PTHrP in amniotic fluid were about 13–16-fold higher than those of PTHrP in either maternal or fetal plasma or in plasma of non-pregnant controls, and equal to or in excess of levels in patients with humoral hypercalcemia of malignancy. Our results on high PTHrP levels in amniotic fluid are in agreement with those reported by Ferguson et al. (14), but we could not confirm their observation that these levels are further increased at term. We have no explanation for this discrepancy. Apparently, the high levels of PTHrP in amniotic fluid do not reach the maternal circulation in amounts that significantly affect circulatory levels or produce a systemic effect (17). Contrary to the finding of Seki et al. (19), we did not detect a higher level of PTHrP in the umbilical artery than in the umbilical vein. This would suggest that the fetus is not a major source of PTHrP in amniotic fluid and that amniotic fluid PTHrP is produced locally in tissues bordering the amniotic sac. Indeed, the results of our in vitro studies support this notion.

In the interpretation of our results on circulatory PTHrP, one should take into account two factors. First, that the sensitivity of the assay is close to the values found in fetal and maternal circulations. Thus, it is possible that the development of more sensitive assays
may reveal small changes in circulatory levels that are of physiological importance but not detectable by present techniques. A second factor is that we do not know the bioactive fragments of the protein in the fetal or maternal circulations. Previous studies have shown that the level of bioactive PTH was three to four times higher than immunoreactive PTH in the umbilical cord plasma (18). By analogy, it is possible that the level of bioactive PTHrP may be higher than the levels of immunoreactive PTHrP measured in our study. In this study, we confirmed the previously reported suppression of PTH levels in cord plasma (18); however, immunoreactive PTHrP levels were also low and therefore cannot account for the high PTH-like bioactivity in umbilical cord blood.

The regulation of expression of the PTHrP gene appears to be affected by different physiological states as well as by a variety of hormones and growth factors. Previous studies demonstrated that corticosteroids and calcitriol suppress PTHrP mRNA levels (20), and that PRL (21), EGF (22) and transforming growth factor beta (13) stimulate PTHrP production. The increased secretion of PTHrP by amniotic fluid cells following stimulation with growth factors and with hormones known to stimulate growth, such as GH, PRL and PL, supports the notion that PTHrP may be a paracrine factor involved in the regulation of cell proliferation and differentiation both in normal and malignant tissues (7, 8, 23). It is of interest that PRL (produced by the decidua) and PL (produced by the placenta), both found

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**Fig. 1.** Immunoreactive parathyroid hormone-related protein (PTHrP) concentrations in amniotic fluid and maternal plasma. Means ± sem are indicated.

**Fig. 2.** Immunoreactive parathyroid hormone-related protein (PTHrP) concentrations in plasma samples from the umbilical cord artery and vein in plasma of newborn babies and of non-pregnant controls. Means ± sem are indicated.
in high concentrations in amniotic fluid, stimulate the formation of PTHrP, which suggests a paracrine relationship. Incidentally, the effect of hPL on the production of PTHrP is one of the rare known manifestations of a possible physiological role for this hormone in fetal cells (24).

The question arises as to the physiological role of PTHrP in the utero-placental unit. Some investigators have suggested that PTHrP, as a potent relaxant of smooth muscle, could be an inhibitor of uterine muscle contraction (12, 14) and a modulator of uterine umbilical and villous vessel tone and blood flow. In
rats, intrauterine occupancy controls the expression of PTHrP in the myometrium. Both PTHrP mRNA and the protein itself reach a maximum towards the end of pregnancy, and decrease sharply post partum (5). A similar pattern also appears in human amnion (14). Furthermore, the PTHrP mRNA was higher in intact than in ruptured membranes (14). It was speculated that down-regulation of PTHrP expression in amnion following rupture of the amniotic sac might play a part in the onset of labor (12, 14).

Parathyroid hormone-related protein is produced in the skin and may be involved in skin differentiation and growth (25, 26). Fetal skin is in direct contact with amniotic fluid and therefore is exposed to high concentrations of PTHrP, which could have an effect on fetal skin differentiation. Because the fetus continuously swallows amniotic fluid, PTHrP may also have a role in fetal intestinal development. Finally, recent studies have indicated that PTHrP increases placental calcium transport in fetal sheep (11, 15). However, it is not clear whether PTHrP has a similar effect on calcium transport in the human placenta. In conclusion, although the normal physiological roles of PTHrP are only beginning to be exposed, accumulating data suggest that PTHrP is involved in the physiology of gestation, in the development of the fetus and placenta and in fetal calcium metabolism.

References


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