Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 8384–8388, September 1992
Physiology

Abundant expression of parathyroid hormone-related protein in human amnion and its association with labor

(pregnancy/myometrium/decidua/placenta/amniotic fluid)

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Communicated by Robert H. Wasserman, June 1, 1992 (received for review December 12, 1991)

ABSTRACT **In animal models, parathyroid hormone-related protein (PTHrP) increases placental calcium transport and inhibits contraction of uterine smooth muscle. The present studies were undertaken to characterize the expression of PTHrP in human uteroplacental tissues. PTHrP mRNA was identified by Northern analysis as a single species (≈1.8 kilobases) in human amnion, chorion, placenta, decidua, and myometrium. The most abundant signal was seen in amnion, where it was 10–400 times that in the other uteroplacental tissues. PTHrP mRNA abundance was decreased in amnion (but not in the other tissues) following the onset of labor ($P < 0.001$). PTHrP mRNA in amnion appeared to be translated to a bioactive peptide, as PTHrP bioactivity and immunoreactive PTHrP in amnion correlated closely with PTHrP mRNA content ($r = 0.86$ and 0.95, respectively; $P < 0.05$ and $P < 0.01$). Amniotic fluid contained PTHrP, 21 ± 6 pmol/liter ($n = 10$) at 16 weeks and 41 ± 9 pmol/liter ($n = 7$) at 38 weeks ($P = 0.05$). These concentrations equaled or exceeded those found in plasma of patients with hypercalcemia secondary to PTHrP. After rupture of the fetal membranes, PTHrP mRNA in amnion was decreased by 78% ($P < 0.0001$). This decrease appeared to be specific for PTHrP mRNA, as glyceraldehyde-3-phosphate dehydrogenase mRNA was unchanged following rupture of membranes. Like PTHrP mRNA, PTHrP bioactivity and immunoreactive PTHrP in amnion decreased significantly following rupture of membranes ($P < 0.03$ and $P < 0.01$, respectively). Since PTHrP is a potent antagonist of uterine muscle contraction, the decrease of PTHrP following rupture of the fetal membranes may play a key role in the onset of labor.**

Parathyroid hormone (PTH)-related protein (PTHrP) was initially purified from human tumors associated with hypercalcemia of malignancy (1). PTHrP and PTH are identical at 8 of the first 13 amino acids, whereas the remainder of the PTHrP molecule shows no similarity to PTH. PTHrP and PTH produce multiple common effects on kidney and bone, and both bind to PTH receptors (2). In humans, circulating concentrations of PTHrP are low or undetectable except in patients with humoral hypercalcemia of malignancy (3). The functions of PTHrP in human physiology are unknown. A variety of studies in laboratory animals suggest that it acts as a paracrine or autocrine factor in skin, brain, lactating mammary gland, and uterus (2, 4, 5).

Experimental evidence suggests a role for PTHrP in the uteroplacental unit. An early study showed that perfusion of sheep placenta with PTHrP reestablished the transplacental calcium gradient that was abolished by fetal parathyroidectomy (6). In rats, the PTHrP gene is expressed in the pregnant uterus (4). Expression is dependent upon uterine occupancy and reaches a peak 48 hr prior to parturition (4). A peak in peptide content has also been demonstrated by biological and immunological assays. As PTHrP and PTH have been shown to be vasodilators and to relax smooth muscles, including uterine smooth muscle (7–9), PTHrP may play a role in (*i*) vasodilation in the uteroplacental unit, (*ii*) expansion of the uterus to accommodate fetal growth, (*iii*) maintenance of the pregnant uterus in a quiescent, relaxed state prior to the onset of labor, and/or (*iv*) relaxation of the uterine cervix to allow passage of the fetus during labor. No studies, however, have explored the expression or role of PTHrP in human uterus or placenta.

The anatomy and the parturition signals of the human uteroplacental unit are different from those of most laboratory animals. Among these differences is the importance of the human amnion, a structure that cannot be easily studied in small rodents. The human amnion is a major site of synthesis of at least two regulatory factors, endothelin (10), a potent vasoconstrictor, and prostaglandin E_2, a stimulator of uterine smooth muscle contraction (11, 12). The present studies suggest an important role of the amniotic membrane in humans as a source of PTHrP.

MATERIALS AND METHODS

Collection of Human Tissues. Myometrium, decidua, chorion, amnion, and placenta (Fig. 1) were obtained from women who required cesarean sections. The protocol was approved by the University of Virginia Human Investigation Committee. The decision that cesarean section was required was made by each patient's primary physician. Only after it was decided that surgery was indicated were the women approached to seek consent for this study. In each instance, a low transverse cesarean section was performed. Following delivery, a thin strip of myometrium, measuring 0.5 × 0.5 × 1 cm, was carefully dissected from the superior aspect of the uterine incision. Care was taken to separate and exclude the serosa as well as the decidua. A decidual biopsy specimen, measuring 0.2 × 0.2 × 1 cm, was obtained similarly. Following delivery of the placenta, a 1-cm^3 segment was excised from the maternal surface. The reflected amnion (Fig. 1) was carefully dissected from the chorion; chorion samples were visually free of adherent decidua. In a separate set of samples, amnion over placenta, as well as reflected amnion (Fig. 1), was removed from underlying tissues, and a portion measuring 2 × 2 cm was used for analysis. Tissues were handled aseptically and with care to avoid ribonucleases. They were immediately placed on previously labeled (and

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Abbreviations: PTH, parathyroid hormone; PTHrP, PTH-related protein; ROS, rat osteosarcoma; IRMA, immunoradiometric assay.

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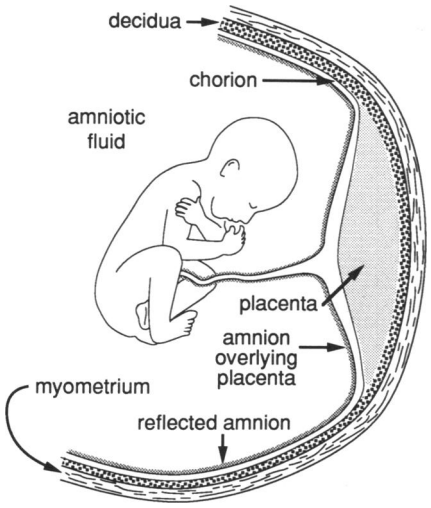

FIG. 1. Uteroplacental unit. Tissues were excised as labeled: myometrium, decidua, chorion, reflected amnion, amnion overlying placenta, and placenta.

autoclaved) slides and transported to the laboratory on dry ice. Tissues were stored at −70°C prior to analysis.

Amniotic Fluid Collection. Amniotic fluid was obtained from a group of women undergoing genetic amniocentesis for advanced maternal age and from some of the previously mentioned group who required cesarean section for delivery. The technique for obtaining amniotic fluid at the time of genetic amniocentesis has been described (13). Amniotic fluid (1 ml) was placed in tubes (containing protease inhibitors: 500 units of aprotinin, 2.5 μg of leupeptin, 2.5 μg of pepstatin, and 100 μmol of EDTA) on ice; the tubes were centrifuged at 2000 × *g* for 10 min, and the supernatants were stored frozen at −70°C. Term amniotic fluid was collected at the time of cesarean section by incising the myometrium and decidua, placing a 20-gauge needle through the directly visualized bulging amniochorionic membranes, and aspirating 1 ml of fluid prior to rupture of the membranes and delivery of the fetus. The validity of the assay for use with amniotic fluid samples was verified by recovery and parallelism studies.

Mice. Timed-pregnant, neonatal, and postweaned female mice (Institute of Cancer Research) were obtained from Dominion Laboratories (Dublin, VA) or Charles River Breeding Laboratories. Pregnant mice were 8 days pregnant on arrival in the laboratory and were killed by cervical dislocation on day 19 of gestation (for pregnancy samples) or during lactation (for mammary gland RNA). Standard laboratory chow and water were freely available and lighting conditions were 12 hr of light and 12 hr of dark. The protocol was approved by the Animal Care Committee of the University of Virginia.

Isolation of RNA and Northern Gel Analysis. Total cellular RNA was isolated by the guanidinium isothiocyanate extraction procedure (14). On each Northern blot, we included lactating mouse mammary gland RNA that had been isolated in the same way and stored in aliquots for this purpose. Before electrophoresis, 20-μg total RNA samples were heated at 50°C for 1 hr in 70% dimethyl sulfoxide with 10% glyoxal. Purified RNA was fractionated by electrophoresis in a 1.5% agarose gel in 0.01 M sodium phosphate buffer (pH 7.0), transferred by capillary blotting to Zetabind (Cuno), and electroblotted. After electroblotting, RNA was bound to Zetabind by exposure to UV light; blots were stored at room temperature. Northern blots were prehybridized at 55°C for 6 hr in Church hybridization buffer (0.5 M sodium phosphate buffer, pH 7.0/1% bovine serum albumin/7% SDS/1 mM EDTA with sonicated, denatured DNA at 100 μg/ml). Hybridization was performed in the same buffer with the labeled cDNA probe BRF 61 (15) added at 3×10^7 cpm/ml. The PTHrP cDNA was obtained from a cDNA library made from RNA of BEN cell origin (a human lung cancer cell line). The cDNA probe was labeled by the random primer hexamer method (BRL/Life Technologies) with [α-^{32}P]dCTP (DuPont/NEN). Blots were washed twice at 55°C, for 30 min each. Hybridization was detected by autoradiography (Kodak X-Omat AR film) at −70°C. PTHrP mRNA was quantified by computer-aided densitometry. Prior to studies of human tissues, we verified the use of the PTHrP cDNA in Northern gel analysis of mouse RNA. In a wide variety of tissues from immature, mature, pregnant, and lactating mice, the most abundant PTHrP mRNA expression was found in the lactating mammary gland as an apparently single message of ≈1.8 kilobases (kb). To adjust for differences in exposure times and specific activities of probes among Northern blots, an aliquot of an RNA preparation from lactating mouse mammary was included on each blot and assigned a value of 1.0. During the course of these studies, two preparations of RNA from lactating mouse mammary gland were prepared and stored in aliquots; PTHrP mRNA abundances in the two preparations agreed within 10% when they analyzed on the same blot.

Bioassay and Immunoassay. Tissues were extracted (5) and assayed for PTHrP bioactivity with the PTH-sensitive rat osteosarcoma (ROS) assay, which measures conversion of [^{3}H]ATP to [^{3}H]cAMP (5). PTHrP immunoreactivity was examined in a two-site immunoradiometric assay (IRMA) for PTHrP-(1–74), as previously reported (3). A modification (16) of the IRMA was used to measure PTHrP in amniotic fluid; the detection limit of the modified assay was 0.1 pmol/liter, and the interassay coefficient of variation was 12% at 0.2 pmol/liter (16).

Statistics. Significance of differences between means was assessed by unpaired Student *t* test except as noted. Results are presented as means ± SEM.

RESULTS

Samples of human pregnant uterus at different stages of labor were examined. An abundant signal was observed in a full-section uterine biopsy sample (containing both myometrium and decidua) from a term (41 weeks) laboring (18 hr) woman (Fig. 2, lane C). In subsequent cases, the decidua and myometrium were dissected and analyzed separately. Although the decidua was clearly positive, the myometrium contained a lesser signal. (See examples in Fig. 2.) Placental samples also had trace signals. The apparent message size (≈1.8 kb) in the human tissues was the same as that found in mouse lactating mammary gland. Northern analysis of the two fetal membranes, amnion and chorion, revealed low or undetectable signals in chorion but abundant PTHrP mRNA in reflected amnion (see below).

To examine whether PTHrP gene expression in the uteroplacental unit is regulated during labor, we obtained reflected amnion, chorion, myometrium, decidua, and placenta from a larger number of laboring and nonlaboring term patients at the time of cesarean section. Fig. 3 summarizes the results. The nonlabor reflected amnion contained the highest PTHrP mRNA signal in the uteroplacental unit, 10–400 times that in other tissues of the human uteroplacental unit. In laboring patients ($n = 9$, Fig. 3), mean PTHrP mRNA in amnion was decreased by 68% ($P < 0.001$). This significant decrease was apparent also when PTHrP mRNA was normalized to 18S RNA (3.4 ± 0.7 vs. 1.6 ± 0.3, $n = 5$ and 8 for nonlabor and labor samples, respectively). Decidua contained 1/10 the PTHrP mRNA seen in reflected amnion (Fig. 3), with no statistically significant difference between the laboring (0.22

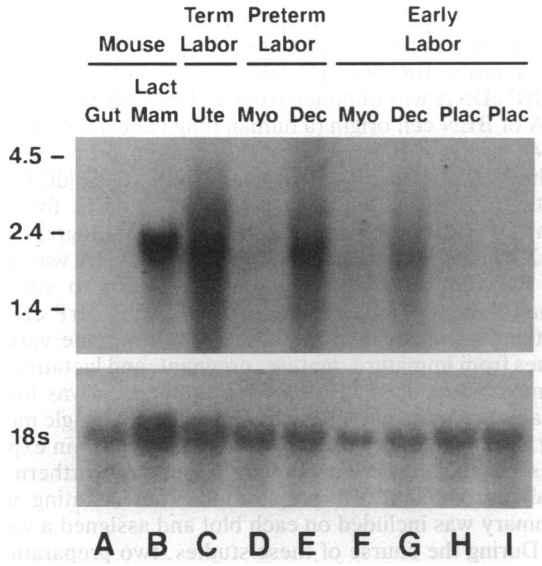

FIG. 2. (*Upper*) Northern blot of RNA extracted from pregnant human uterus and probed with human PTHrP cDNA. Each sample represents 20 μg of total RNA. Lanes: A, mouse small intestine; B, lactating mouse mammary gland; C, uterus from patient (term) after 18 hr of labor; D, myometrium from preterm patient; E, decidua (endometrium) from preterm patient; F, myometrium from term patient in early labor; G, decidua (endometrium) from term patient in early labor; H and I, placentas from term patients. Size markers are in kilobases. (*Lower*) Northern blot of the same samples of RNA extracted from pregnant human uterus was used to detect 18S RNA in the same samples.

± 0.13) and the nonlaboring (0.30 ± 0.16) states. Similarly, no statistically significant effect of labor was seen on the less abundant expressions of PTHrP observed in chorion, myometrium, and placenta (Fig. 3).

In view of previous evidence (17, 18) that PTHrP expression in uterus and urinary bladder is increased by stretch (i.e., by uterine occupancy with a fetus or balloon or by the bladder filling with urine), we examined PTHrP mRNA abundance in amnion from intact fetal membranes (i.e., sacs filled with amniotic fluid) and membranes that had ruptured and lost fluid prior to cesarean section. The expression of PTHrP showed a striking relationship to occupancy of the amniotic sac by its fluid contents (Fig. 4). PTHrP mRNA abundance

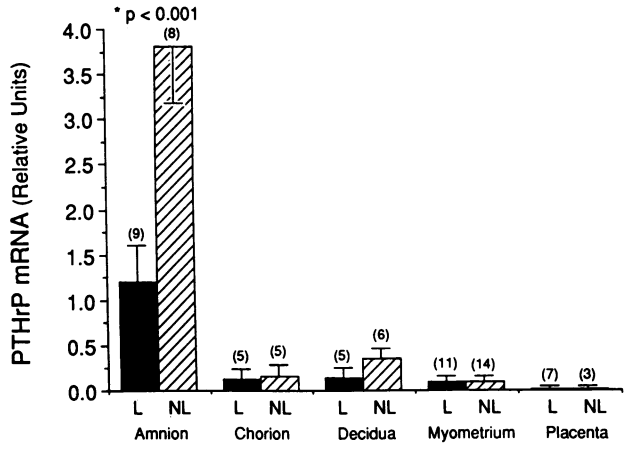

FIG. 3. PTHrP mRNA abundance in tissues of the uteroplacental unit from laboring (L) and nonlaboring (NL) patients. PTHrP mRNA abundance was assessed by Northern analyses and expressed in relative units as multiples of the PTHrP mRNA in lactating mouse mammary gland. Numbers in parentheses indicate numbers of individual patients in each group.

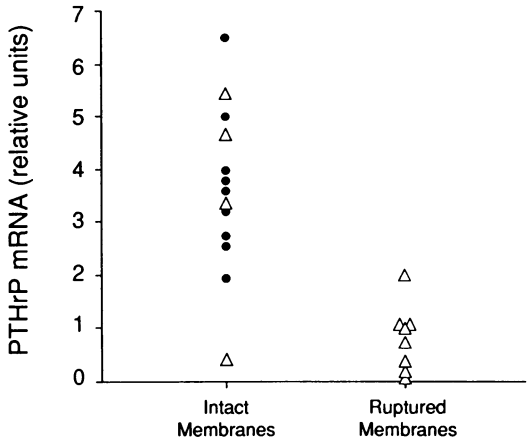

FIG. 4. PTHrP mRNA abundance in reflected amnion from intact and ruptured fetal membranes. Results (by Northern analysis) are expressed in arbitrary units relative to the signal in lactating mouse mammary gland RNA. △, Labor; ●, nonlabor.

in amnion from intact (filled) sacs was 3.6 ± 0.4 relative units but decreased by 78% to only 0.8 ± 0.2 unit for amnion from which the fluid contents had drained ($P < 0.0001$). Among 13 amnions from intact membranes (Fig. 4), the mean PTHrP mRNA abundance was the same (3.5 relative units) in amnions from nine nonlaboring and four laboring women, suggesting that membrane status rather than labor is the key regulator of PTHrP expression. In all but one case with ruptured membranes, the rupture occurred spontaneously; in amnion from the case with artificially ruptured membranes, PTHrP was within one SEM for the samples from spontaneously ruptured membranes.

The decrease of PTHrP mRNA in amnion following rupture of membranes appeared to be specific, as phosphoglyceraldehyde dehydrogenase (PGAD) mRNA (Fig. 5) did not change significantly with rupture of membranes and onset of labor ($P > 0.1$), and the ratio of PTHrP to PGAD mRNA decreased ($P < 0.025$) from 2.0 ± 0.5 ($n = 3$) to 0.26 ± 0.17 ($n = 3$) in ruptured membranes (Fig. 5). The decrease of PTHrP mRNA appeared to occur rapidly following rupture of membranes; in three amnions obtained within 4 hr of rupture, the mean PTHrP mRNA abundance was only 5% of that found in samples from intact membranes. As expected, the PTHrP mRNA difference between intact and ruptured mem-

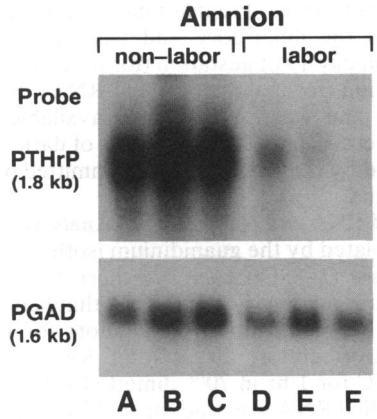

FIG. 5. Northern analysis of amnion (20 μg of total RNA per lane) from nonlaboring patients with intact fetal membranes (lanes A–C) and laboring (7–12 hr) patients with ruptured (4–11 hr) membranes (lanes D–F). PTHrP cDNA (*Upper*) and phosphoglyceraldehyde dehydrogenase (PGAD) control cDNA (*Lower*) probes were used. Durations of labor were 12, 9, and 7 hr (lanes D–F), respectively, and the durations of ruptured membranes were 11, 4, and 8 hr.

branes was not changed by normalizing the PTHrP mRNA data to 18S RNA (e.g., 3.4 ± 0.5 vs. 1.3 ± 0.3, $P < 0.01$, for three intact and three ruptured samples analyzed on the same Northern blot).

Tissue extracts of reflected amnion were assayed for PTHrP bioactivity in the ROS adenylate cyclase assay and for PTHrP immunoreactivity in a two-site IRMA. As shown in Fig. 6, PTHrP immunoreactivity and PTHrP bioactivity were higher in amnion from nonlaboring patients with intact membranes than in amnion from laboring patients with ruptured membranes ($P < 0.01$ and $P < 0.03$, respectively, for immunoreactivity and bioassay), and both immunoreactivity and bioactivity were highly correlated with PTHrP mRNA expression (bioactivity; $r = 0.86$ and $P < 0.05$; immunoreactivity; $r = 0.95$ and $P < 0.01$).

The regional distribution of PTHrP mRNA in the amnion was studied by carefully dissecting amnion from the fetal surface of the placenta ("amnion overlying placenta") and comparing it, by Northern analysis, with paired samples of reflected amnion. (For anatomical locations, see Fig. 1.) Representative results are shown in Fig. 7. In seven paired samples (four from laboring women and three from nonlaboring women), PTHrP mRNA was consistently (1.4- to 5.5-fold) higher in amnion overlying placenta (amnion P in Fig. 7) than in reflected amnion (4.5 ± 0.6 vs. 1.9 ± 0.6 density units, $P < 0.001$, paired t test).

In view of the large PTHrP mRNA signal in amnion, we measured immunoreactive PTHrP in amniotic fluid. Amniotic fluid was examined at two gestational ages, 16 weeks and 38–40 weeks. Immunoreactive PTHrP was present in high concentrations at both gestational ages; mean values (±SEM) were 21 ± 6 pmol/liter ($n = 10$) at 16 weeks and 41 ± 9 pmol/liter ($n = 7$) at 38 weeks ($P = 0.05$).

DISCUSSION

These studies demonstrate the abundant, regulated expression of PTHrP in human amnion and high concentrations of the hormone in amniotic fluid. The PTHrP mRNA abundance in human amnion was 10–400 times higher than that in other tissues of the uteroplacental unit (Fig. 3). The mean concentrations of PTHrP in amniotic fluids from second- and third-trimester pregnancies (21 and 41 pmol/liter) equal or exceed

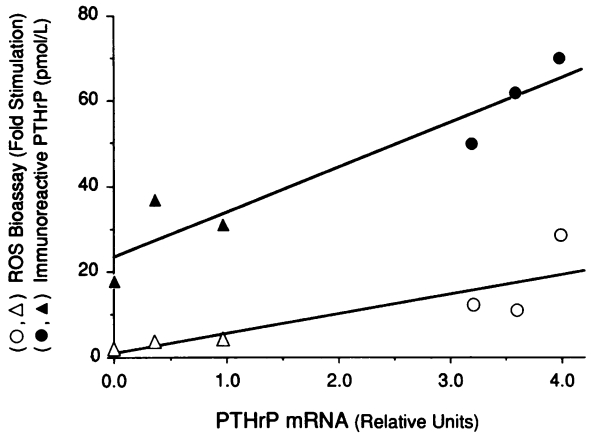

FIG. 6. Correlation of immunoassayable PTHrP (filled symbols) and bioassayable PTHrP (open symbols) in tissue extracts with PTHrP mRNA in samples of reflected amnion from laboring patients with ruptured membranes (triangles) and nonlaboring patients with intact membranes (circles). Bioactivity is expressed as fold stimulation over basal in the ROS assay. Basal in this assay was 199 cpm. Immunoreactivity is expressed as pM equivalents of PTHrP-(1–74) in tissue extracts. The mean protein concentration in tissue extracts was 2.29 ± 0.12 mg/ml. The amount of PTHrP mRNA (1.8 kb) was calculated as relative units as described in *Materials and Methods*.

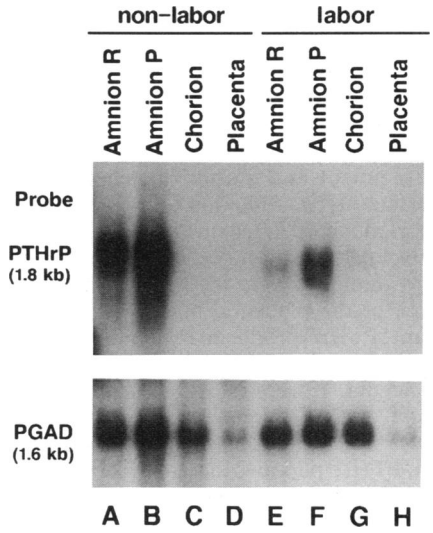

FIG. 7. Representative Northern analysis of PTHrP mRNA in reflected (R) amnion (lanes A and E), amnion overlying placenta (P) (lanes B and F), chorion (lanes C and G), and placenta (lanes D and H). Lanes A–D, nonlaboring patient; lanes E–H, laboring (4 hr) patient. Phosphoglyceraldehyde dehydrogenase (PGAD) mRNA was probed as a transcription control.

the mean concentrations of PTHrP in the systemic circulation of patients with humoral hypercalcemia of malignancy (3) and exceed those found in human plasma under reported physiologic conditions.

The finding of abundant synthesis of PTHrP mRNA in the human amnion was unexpected. In the rat uteroplacental model, reported expression of uterine PTHrP has been confined to the uterus (4). Similarly, in our studies of the mouse uteroplacental unit, we found no PTHrP mRNA synthesis in fetal membranes (data not shown). The chorion–decidua and placenta are key sites of synthesis and secretion of peptide and steroid hormones during pregnancy. By contrast, the human amnion is avascular and not considered a major site of peptide hormone synthesis and secretion, with the exception of endothelin (10). Consistent with our findings, however, is the finding (19) of immunoreactive PTHrP in human fetal membranes (combined amnion and chorion).

The source of PTHrP in amniotic fluid is not defined with certainty by these studies. Two potential sources are fetal urine and amnion. At term, approximately half of the volume of amniotic fluid is fetal urine. In one sample of fetal urine that became available during the course of these studies, PTHrP was undetectable while PTHrP in the amniotic fluid was at the expected high concentration (unpublished). Thus, we favor the interpretation that PTHrP in amniotic fluid comes from amnion. If correct, this conclusion suggests that amnion makes and secretes PTHrP at least as early as 16 weeks.

The accumulation of PTHrP in amniotic fluid suggests potential target tissues for the hormone in the fetus. Because the fetus swallows amniotic fluid, PTHrP has direct access to the fetal gastrointestinal mucosa, which is thus a potential target tissue; in addition, because peptides can be absorbed intact through fetal intestinal epithelium, the hormone has the potential to act systemically on the fetus (and placenta). As fetal breathing transports amniotic fluid to the respiratory tract, PTHrP may act on pulmonary epithelium; a recent immunohistochemical study demonstrated immunoreactive PTHrP in epithelia of the human fetal lung (20). Fetal skin is in direct contact with amniotic fluid and thus is exposed to high concentrations of PTHrP; studies suggest that PTHrP plays a role in normal skin physiology (2).

The anatomy of the human amnion suggests other target tissues for amniotic PTHrP. As shown in Fig. 1, the human

amnion not only forms a sac that contains the fluid in which the fetus resides, the amnion also (*i*) is juxtaposed to the maternal uterus, separated from it only by the thin chorionic membrane, (*ii*) covers the fetal surface of the placenta, and (*iii*) covers the umbilical vessels (21). Thus production of hormones (e.g., endothelin, prostaglandins) by the fetal membranes (amnion and chorion) appears to provide a mechanism to affect uterine function (10–12). This may be especially important for PTHrP, which is a potent inhibitor of uterine muscle contraction (9).

Since the amnion covers the vessels that course over the fetal surface of the placenta, and since PTHrP is a potent vasorelaxant, PTHrP produced by amnion cells overlying the placenta has the potential to affect fetal blood flow to and from the placenta. Moreover, PTHrP from amnion may affect the placenta itself, as human placenta contains a PTH (PTHrP) receptor located on the fetal basal membrane (22). In addition, PTHrP peptides increase placental calcium transport in animal models, apparently by additional mechanisms independent of the PTH receptor (23). Thus, human placenta is an important candidate target tissue for PTHrP.

Decreased amniotic expression of PTHrP at the time of membrane rupture appears to be a part of the coordinated set of molecular and cellular changes associated with labor. These changes include the appearance of oxytocin receptors in myometrium and decidua, the formation of gap junctions in myometrium, and the production of prostaglandins in decidua and amnion (24). In certain species (e.g., sheep) abrupt changes in circulating cortisol, estrogen, and progesterone appear to play important roles in the onset of labor (24). In humans, however, the signal(s) that initiates labor is unknown.

The present studies do not define the mechanism of PTHrP down-regulation in amnion after rupture of the membranes, but studies of other organs appear to be relevant. The amniotic sac must expand or stretch to accommodate the growing fetus and the amniotic fluid. In two rat organs, uterus and urinary bladder, increased internal pressure [from fetuses (or a balloon) or urine, respectively] produces increased PTHrP expression in the wall of the hollow viscus (17, 18); the increased expression of PTHrP is reversed upon relief of the pressure (e.g., by deflating a balloon within the uterus) and can be prevented by blocking the stretch (e.g., by banding the urinary bladder). (Note that the effect of PTHrP to relax smooth muscle contrasts with the tendency to contraction of isolated smooth muscle subjected to stretch.) By analogy to these organs, internal pressure on the amnion during pregnancy may be expected to sustain the production of PTHrP. During these periods, PTHrP would be available to relax the uterus, allowing it to expand to accommodate the growing fetus. By contrast, during labor, the internal pressure of the amniotic fluid is countered by the force of uterine contractions; similarly, upon rupture of the amniotic sac, with the loss of amniotic fluid, the amniotic sac collapses. Either process may be expected to lead to decreased expression of PTHrP. Although the mechanism(s) that induces human labor is unknown, spontaneous and induced ruptures of the membranes are known to induce uterine contractions. As rupture of membranes decreased PTHrP mRNA abundance (Fig. 4), it is tempting to speculate that down-regulation of PTHrP expression in amnion may play either a primary or a permissive role in the onset of labor.

We gratefully acknowledge Dr. M. R. Pandian (Nichols Institute, San Juan Capistrano, CA) for assays of immunoreactive PTHrP in amniotic fluid; Dr. K. L. Insogna for performing the ROS assay; and Dr. K. Benirschke for helpful discussion of the anatomy of the amnion and review of Fig. 1. This research was supported by National Institutes of Health Grant HD12335.

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