High Levels of Intrauterine Corticotropin-Releasing Hormone, Urocortin, Tryptase, and Interleukin-8 in Spontaneous Abortions

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Stress induces CRH secretion that activates hypothalamic-pituitary-adrenal axis and is also abortogenic. In addition to hypothalamus, CRH and its analog urocortin (Ucn) are also secreted locally outside the brain where they activate mast cells leading to inflammation; however, the level of CRH and Ucn or mast cell mediators has not been examined in products of conception (POC). CRH and Ucn were measured by enzyme immunoassay, tryptase by fluoroenzyme immunoassay, and IL-8 by ELISA in POC of 7-9 wk gestation from Caucasian women; they were divided into group I with elective abortions (n = 4), group II with one spontaneous abortion (n = 12), and group III with at least two spontaneous abortions (n = 7). CRH, Ucn, tryptase, and IL-8 levels were higher (P < 0.05) in group III (8683 ± 1201 pg/g, 7961 ± 1499 pg/g, 1553 ± 572 ng/g, and 8317 ± 1874 pg/g, respectively) than group II (2561 ± 314 pg/g, 2349 ± 394 pg/g, 463 ± 97 ng/g, and 3199 ± 449 pg/g, respectively) and group I (163 ± 162 pg/g, 328 ± 327 pg/g, 72 ± 31 ng/g, and 3681 ± 931 pg/g, respectively). Immunostaining of POC showed significantly more tryptase in group III women. High POC levels of CRH and Ucn under stress in habitual spontaneous abortions may activate uterine mast cells to secrete abortogenic tryptase and IL-8. (Endocrinology 144: 2285–2290, 2003)

A NUMBER OF studies have reported increased risk of pregnancy loss associated with psychological stress (1). Moreover, the decidua of women with high levels of stress had significantly higher number of tryptase, CD8, as well as TNF-α positive cells (1). Also, women who miscarried were more likely to have experienced severe stress in the 3 months preceding, as well as in the fortnight immediately before miscarriage (2). Maternal stress due to environmental and/or socioeconomic factors (e.g. poverty, unmarried status, as well as loss of employment, housing, partner) were also linked to preterm delivery (3). It is well known that stress activates the secretion of CRH primarily from the hypothalamus, leading to stimulation of the hypothalamic-pituitary-adrenal axis. Recent evidence suggests that the human endometrium is a neuroendocrine organ, able to produce several brain peptides, including CRH (4).

The placenta and intrauterine tissues produce increasing amounts of CRH during pregnancy, with corresponding rises in plasma levels at term. Women with preterm labor, or those destined to have premature delivery, have higher mid-gestation plasma CRH than those who deliver at term (5). The most marked elevations in maternal plasma CRH have been reported in preeclampsia (1, 6, 7). It is thought that CRH enhances prostaglandin production in uterine tissues, leading to parturition (3). In contrast, urocortin (Ucn) is structurally related to CRH; its plasma levels are highest only during labor, but still low compared with CRH (8). Ucn was detectable in maternal plasma from 7 wk gestation, but the concentration did not change as gestation progressed (9). Ucn is expressed in ovary, placenta, fetal membranes, and pregnant uterine tissues (such as endometrial epithelial and stromal cells, myometrium, and vascular smooth muscle cells), but not in nonpregnant uterus (4). Human placenta and gestation-related tissues (amnion, chorion, decidua) express Ucn mRNA and contain immunoreactive Ucn (10). Based on the staining intensity for immunoreactive Ucn, decidua may be the main site of Ucn production (11). Ucn stimulates placental secretion of ACTH and prostaglandin E2, and it also modulates myometrial contractility (10).

The endometrium, myometrium, and outer decidua contain mast cells that are increased by more than 10-fold in decidua in abortions (12). Mast cells are essential for allergies and inflammation by releasing histamine, proteases (tryptase, chymase), proteoglycans, prostaglandin D2, leukotriene C4, and several multifunctional cytokines including IL-8 (13). Tryptase is a tetrameric serine proteinase that constitutes 20% of the total mast cell protein; it is stored in the secretory granules in a fully active form and released together with other preformed mediators during mast cell degranulation (14). Tryptase may act to amplify mast cell activation and/or induce microvascular leakage (15). Endometrium also produces IL-8 that is abortogenic (16). Endo-

Abbreviations: APAAP, Alkaline phosphatase antialkaline phosphatase; POC, products of conception; RT, room temperature; SP, substance P; Ucn, urocortin.

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metrial CRH, IL-6, IL-1, and prostaglandins participate in local inflammatory phenomena associated with decidualization of endometrial stroma and the implantation of the fertilized eggs (17). We investigated the levels of CRH, Ucn, tryptase (including immunocytochemistry), and IL-8 in POC from women with elective or spontaneous abortions. High levels of CRH, Ucn, tryptase, and IL-8 were detected in POC from women with habitual spontaneous abortions.

Materials and Methods

**Tissue collection and extract preparation**

POC-containing myometrium, fetal membranes (amnion and chorion), and chorionic villi were obtained from Caucasian women (17–43 yr old) of Greek ethnic background with spontaneous and elective (termination of unwanted pregnancy) abortions of 7–9 wk gestation established by menstrual history confirmed by ultrasonography. These subjects were middle class with 6 ± 2 yr of postelementary education; they had no history of chromosomal or hormonal abnormalities, genetic defects, structural uterine abnormalities, antithrombophilic syndromes, or infections. POC samples had no labeling that could identify the subjects and were analyzed by investigators blinded to the abortion history of the donor. Subjects were divided into group I (17–23 yr old) with elective abortions (n = 4), group II (19–29 yr old) with one spontaneous abortion (n = 12), and group III (21–43 yr old) with at least two (habitual) spontaneous abortions (n = 7); the average number of spontaneous abortions in the latter group was three. This investigation was approved by the Scientific Review Committee of the InterBalkan Medical Center. Immediately after collection, all samples were frozen and stored at −80 C until extract preparation. For the assays, samples were thawed and weighed; they were then placed in 1 ml of 0.1 m acetic acid in polypropylene tubes and boiled for 10 min. After cooling on ice, the samples were homogenized (Polytron, Kinematica, Kriens-Lucerne, Switzerland) and the homogenates were centrifuged at 13,000 × g for 15 min at 4 C. The supernatant fluids were collected and stored at −80 C until CRH, Ucn, tryptase, or IL-8 could be assayed.

**CRH, Ucn, tryptase, and IL-8 assays**

CRH and Ucn were assayed using an Enzyme Immunoassay (EIA) Kit (Phoenix Pharmaceuticals, Inc., Belmont, CA) as per directions of the manufacturer (18). The concentration range for both CRH and Ucn that is detectable by the kit is 0–100 ng/ml. Briefly, the samples were treated with biotinylated rabbit anti-CRH (human) serum or rabbit anti-Ucn (human) serum and were incubated for 2 h at room temperature (RT). After the wells were washed, streptavidin-horseradish peroxidase was added into the wells and further incubated for 1 h at RT. The wells were washed, and the substrate 3,3',5,5'-tetramethylbenzidine (TMB) solution was added and incubated for 1 h at RT. The reaction was then stopped by adding 2 n HCl, and the optical density was read (MR 600, Dynatech Corp., Chantilly, VA). Tryptase was measured using a Unicap 100 automated unit (Pharmacia & Upjohn, Inc., Uppsala, Sweden) by fluoroenzyme-immunoassay (19). The minimum detectable level of tryptase is 1 ng/ml. IL-8 was measured by ELISA (R&D Systems, Minneapolis, MN) with a minimum detectable level of 10 pg/ml.

**Immunohistochemical staining for tryptase**

Immunostaining for tryptase was performed as reported previously (20). Briefly, cryostat sections (8 μm) were prepared, air dried, and stored at −80 C until staining. Sections were air-dried for about 1 h at RT and fixed with Carnoy’s solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 3 min. The sections were then stained for mast cell tryptase by the alkaline phosphatase antialkaline phosphatase (APAAP) procedure using the DAKO APAAP Kit system (DAKO Corp., Carpinteria, CA). The sections were incubated overnight at 4 C with mouse antihuman tryptase monoclonal antibody (Chemicon, Temecula, CA) diluted to working dilutions of 1 μg/ml in Tris-HCl-PBS (pH 7.6), plus 10% fetal bovine serum. The sections were then brought to RT and incubated with rabbit antiserum (Ig fraction) to mouse Ig for 30 min. The samples were then incubated with the APAAP immune complex for 30 min. Between each incubation, sections were rinsed in Tris-buffered saline (pH 7.6) for 10 min. The reaction was finally developed with substrate solution (naphthol AS-MX phosphate, Fast Red, and levamisole) for 20 min and then rinsed briefly in a water bath. Negative controls were performed either by the omission of the primary antibody or by using an isotype-matched mouse IgG1 antibody instead of the primary antibody. Positive staining resulted in the formation of a bright red precipitate at the site of the target antigen tryptase.

**Statistical analysis**

All results were analyzed using ANOVA followed by Tukey’s multiple comparison test. Each value represents the mean of duplicate assays. The level of statistical significance was set at a P value less than 0.05.

**Results**

**CRH, Ucn, tryptase, and IL-8 levels**

CRH, Ucn, tryptase, and IL-8 levels were increased in most samples from women with spontaneous abortions when compared with the levels in elective abortions. CRH (Fig. 1A), Ucn (Fig. 1B), tryptase (Fig. 1C), and IL-8 (Fig. 1D) levels were significantly (P < 0.05) higher in group III women with at least two spontaneous abortions (8683 ± 1201 pg/g; 7961 ± 1499 pg/g; 1553 ± 572 pg/g; and 8317 ± 1874 pg/g, respectively) than in group II women with one spontaneous abortion (2561 ± 314 pg/g; 2349 ± 394 pg/g; 403 ± 97 pg/g; and 3199 ± 449 pg/g, respectively). CRH, Ucn, and tryptase, but not IL-8, were also significantly higher than in group I women with elective abortions (163 ± 162 pg/g; 328 ± 327 pg/g; 72 ± 31 ng/g; and 3681 ± 931 pg/g, respectively). There was no significant difference of CRH, Ucn, and tryptase levels among group I and group II women.

**Immunohistochemical staining for tryptase**

POC from group III women with at least two spontaneous abortions showed strong positive staining for tryptase (Fig. 2A) compared with faint staining in those POC from group II (Fig. 2B). POC from group I had negligible staining (data not shown). Control sections (without primary antibody treatment) did not show any positive staining reaction (Fig. 2C).

**Discussion**

CRH, Ucn, tryptase, and IL-8 levels were significantly increased in POC of women with spontaneous abortions, and these levels were highest in patients with at least two spontaneous abortions (average = 3). The only condition in humans in which CRH is detectable in plasma is pregnancy, and it is mainly placental in origin (21, 22), although pregnant uterine tissue also produces CRH (8, 9, 12, 17, 21). The earliest plasma CRH increase was reported to be no more than 23.2 ± 1.3 pg/ml at 8 wk gestation (5). During labor, plasma CRH levels increase, and women with preterm labor or those destined to have premature delivery show higher CRH during the second trimester than those who deliver at term (5, 23). High plasma maternal CRH was reported during miscarriage, abnormal pregnancies, or preterm labor or pre-eclampsia (1, 6, 7, 24, 25). Pregnancies with intrauterine growth retardation and/or hypertensive disorders had elevated plasma CRH levels (25). Umbilical cord CRH was also elevated in fetuses with fetal growth restriction (26). Nevertheless, there was no detectable plasma CRH at 8 wk ges-
tation, and there was no relationship between any increased plasma CRH in eight cases of early pregnancy loss (5). Ovary, placenta, fetal membranes and pregnant uterine tissues (such as endometrial epithelial and stromal cells, myometrium, and vascular smooth muscle cells), but not the nonpregnant uterus, express Ucn (4). Human placenta, amnion, chorion, and decidua also express Ucn mRNA and contain immuno-reactive Ucn (10). However, in contrast to CRH, Ucn plasma levels increase during labor, but not during pregnancy (8). Ucn was detectable in maternal plasma from 7 wk gestation, but the concentration did not change as gestation progressed (9). Ucn induces placental secretion of ACTH and prostaglandin E₂ and modulates myometrial contractility (10).

There are no available data on CRH levels in normal uterus at 6–9 wk gestation; however, normal and tumor epithelial cells of human endometrium have been reported to produce CRH (17). The levels of CRH (8683 ± 1201 pg/g) and Ucn (7961 ± 1499 pg/g) detected in the POC from women with at least two spontaneous abortions (group III) are higher than the CRH values reported for normal placenta (1904 ± 489 pg/g), fetal membranes (645 ± 155 pg/g), or even pre-eclamptic placenta (5897 ± 1576 pg/g; Ref. 23); no Ucn was detected in the latter study. Circulating CRH levels were shown to increase from 23.2 ± 1.3 pg/ml at 8 wk to 34.3 ± 2.2 pg/ml at 16 wk and 1294 ± 113 pg/ml at term (5); these latter values were not considered suggestive of possible early

**FIG. 1.** Scattergrams showing CRH, Ucn, tryptase, and IL-8 levels in POC from individual women with elective abortions (group I), one spontaneous abortion (group II), and at least two spontaneous abortions (group III). CRH (A), Ucn (B), tryptase (C), and IL-8 (D) levels were significantly (P < 0.05) higher in group III women than women in groups II and I, except between groups III and I for IL-8.
FIG. 2. Immunohistochemical staining for mast cell tryptase in aborted POC. Group III women with at least two spontaneous abortions (A) showed stronger positive staining (bright red) for tryptase than women in groups II (B) and I (not shown). Control (C) samples without primary antibody treatment did not show any positive staining. Magnification, ×100.
pregnancy loss (5). Another study reported that the plasma CRH at 26 wk gestation was 34.7 ± 27.0 pm, but an increase to more than 90 pm was considered predictive of preterm delivery (27).

We report here high level of tryptase in POC from women with at least three spontaneous abortions. This finding is supported by the high number of tryptase-positive uterine mast cells previously reported in decidua of abortions (12). These mast cells could be activated by CRH and Ucn secreted locally under stress. We previously reported that both CRH and Ucn cause skin mast cell degranulation (28, 29). It was recently shown that mast cell tryptase cleaves proteinase-activated receptor 2 and induces widespread inflammation, partially through the release of proinflammatory neuropeptide substance P (SP; Ref. 30). A large proportion of primary spinal afferent neurons express proteinase-activated receptor 2 and contain SP. Mast cells from a variety of sites, such as the urinary bladder (31), are closely associated with SP-positive neurons and respond to SP with histamine release and cytokine production (32). This new mechanism of proteinase-induced neurogenic inflammation may contribute to stress-induced proinflammatory effects of mast cells in aborted POC (33). Inflammatory cytokines, such as IL-8, may play an important role in these processes leading to labor or abortions (34) by recruiting neutrophils and lymphocytes in the endometrium (16). A previous study reported that women with spontaneous abortions had significantly decreased plasma levels of IL-8, IL-6, and IL-11 compared with those with normal pregnancies (35); nonpregnant women had no detectable plasma cytokine levels (35). The high IL-8 level we report, in POC from group III women may be due to the release of IL-8 from the endometrium (16, 36), as well as from an increased number of resident mast cells that are degranulated in abortions, as reported previously (12). Uterine mast cells degranulate after stress exposure of pregnant mice, possibly leading to release of IL-8 and TNF-α that could be involved in abortions (29). Acute stress has also been shown to activate mast cells in the human and rat bladder (37).

CRH and Ucn are released under stress mainly from the hypothalamus to activate the hypothalamic pituitary-adrenal axis, and also outside the CNS where they can have proinflammatory effects (1, 38). Stress can induce preterm delivery and abortions. For instance, 54% of those who miscarried had at least one stressful episode in the preceding 3 months, compared with the 15% reported in the control group, and most had a stressful episode in the fortnight immediately preceding miscarriage (39). In another study, spontaneous abortions at about 11 wk gestation were strongly associated with more stressful episodes than other gestational periods (40). A third study reported more than a 2-fold increase in the rise of spontaneous abortions in the presence of psychological stress (41). Stress-associated preterm deliveries reflect CRH expression by placenta, decidua, and fetal membranes, which induce prostaglandin production in these tissues and promote labor (3).

The information provided above strongly argues against systemic CRH being the source of elevated CRH or Ucn in POC. One would, therefore, have to invoke local release without concomitant elevations in plasma CRH/Ucn. For instance, local CRH release was shown in the joints of patients with rheumatoid arthritis (42). Moreover, articular CRH in rheumatoid arthritis was shown to be elevated without systemic elevations in plasma CRH (43). CRH was also elevated in joints of Lewis rats with inflammatory arthritis (44) and in the skin of Sprague-Dawley rats exposed to 120 min of restraint stress (45). Alternatively, plasma CRH may be elevated but rendered inactive and inaccessible to measurement because of its association with CRH-binding protein (46). Sources of local intrauterine CRH/Ucn could be: 1) the placenta or other intrauterine pregnant tissues [as discussed above; Refs. 8–10, 17, 21, 22 (immunohistochemistry has also shown the presence of CRH and Ucn in placenta; Ref. 11)]; 2) local nerve endings (47); 3) immune cells (48, 49); or 4) mast cells (50). The question then arises about what triggers local release of CRH or Ucn. We hypothesized that stress may be a trigger because psychological stress was associated with increased incidence of spontaneous abortions as discussed above. Both maternal psychological stress and/or local fetal stress due to local infection or lack of amniotic fluid could induce the release of CRH and/or Ucn locally in the uterus. For instance, we recently showed that human umbilical cord-derived mast cells contain CRH and Ucn, which they can secrete upon immunological stimulation (50).

In conclusion, POC from women with habitual spontaneous abortions are associated with higher levels of CRH, Ucn, tryptase, and IL-8. We hypothesized that systemic or uterine stressful conditions may increase the incidence of spontaneous abortions by inducing the local release of CRH and Ucn, which then activate endometrial mast cells to secrete abortogenic tryptase and IL-8. Future studies should investigate whether there is any association between the mother’s psychological state, plasma CRH, CRH-binding protein, and Ucn, as well as local POC levels of these hormones.

Acknowledgments

We thank all the medical and nursing staff of Obstetrics and Gynecology, InterBalkan Medical Center, Thessaloniki, Greece, for the collection of POC samples. We also thank Miss Ysahin Tien for her patience and word-processing skills.

Received January 13, 2003. Accepted March 6, 2003.

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This research was supported by Theta Biomedical Consulting and Development Co., Inc. (Brookline, MA).

References

6. Moswad AH, Goldenberg RL, Mercer B, Meis PJ, Iams JD, Das A, Caritis SN,


