The Expression of Corticotropin-Releasing Hormone Gene and its Immunohistochemical Analysis in Human Trophoblast of Normal Pregnancy and Trophoblastic Disease

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Abstract : *Immunoreactive corticotropin-releasing hormone (IR-CRH) in maternal plasma increases progressively with pregnancy and rapidly declines after delivery. So the production site of IR-CRH is thought to be placenta. We studied the expression of CRH gene and its immunohistochemical localization in human developing chorionic tissue, amniotic membranes, uterine myometrium and fresh surgical specimen of hydatidiform mole with the methods of Northern blotting and avidin-biotin complex staining. The results are 1) the CRH mRNA were identified in third trimester and term placentae, but could not be demonstrated in first and second trimesters placentae, 2) the CRH mRNA expresses in amniotic membranes but not in myometrium of normal term pregnancy, 3) IR-CRH localization was demonstrated in the cytotrophoblast of placenta and decidua of first trimester, and in the amniotic membranes of term pregnancy and, 4) the CRH mRNA and its immunohistochemical localization was not detected in trophoblast of hydatidiform mole. These results suggest that the sources of increased IR-CRH in human plasma and amniotic fluid during pregnancy are placenta, decidua and amniotic membranes, and that the gene expression and secretion / storage ratio of placental CRH increases as pregnancy advances. The expression in trophoblast of hydatidiform mole may be suppressed because of the characteristic change of trophoblast. (Thai J Obstet Gynaecol 1991;3: 21-30.)*

Key words: corticotropin-releasing hormone (CRH), northern blotting, immunohistochemical localization

It was formerly supposed that corticotropin-releasing hormone (CRH) exists in the hypothalamus[1,2]. In 1981, ovine-CRH was first purified from ovine hypothalami by Vale et al[3] and its amino acid sequence as well as the primary structure of the biosynthetic precursor of human CRH (hCRH) was

determined[4]. Subsequently, Sasaki et al[5] reported that the concentration of immunoreactive (IR)-CRH in human plasma progressively increased during pregnancy and rapidly declined after delivery, and the production site of the IR-CRH is supposed to be placenta. To date, the expression of hCRH gene in term placenta has been reported[6], but there has been no systemic study at various stages of pregnancy, nor in amniotic membranes, uterine myometrium or trophoblastic disease. In this paper, we studied hCRH in these tissues at both gene and protein levels by the use of molecular biological and immunohistochemical methods.

Materials and Methods

Tissues

Placentae of 6, 10, 14, 15, 17 and 23 weeks of pregnant women were obtained by therapeutic abortion, and that of 28 weeks of pregnancy was obtained from a woman who went into premature delivery. Placentae, amniotic membranes and uterine myometrium of 40 weeks gestation were obtained from women undergoing repeat, or elective cesarean section. Hydatidiform mole, from a patient who was diagnosed at 10 weeks of pregnancy, was sampled as the specimen of trophoblastic disease. All patients gave their informed consent to participate in this study. Hypothalamic and liver explants were obtained from male Sprague Dawley rats (180-200g weight, Japan Animal Farm).

Tissues for hybridization experiments were frozen in liquid nitrogen and stored -80°C until RNA isolation. For immunohistochemical stain, samples of 6 and 40 weeks of pregnancy, and of hydatidiform mole were fixed with 10% formalin /PBS for 48 hours.

Northern blotting

CRH probe

The inserted DNA segment containing the gene for the CRH precursor, which was subcloned into plasmid of pBR 322, was 3.8kb genomic DNA[4]. The 265hp DNA fragment which contains a part of first intron[5] and, part of second exon was separated from Pvull digestion of the plasmid, and used for CRH probe.

Labelling of probe

The probe was labelled with (α-^{32}p) dCTP (3000Ci/mmol/MEN) by multipriming system kit (Boehringer Mannheim). The specific activity of the radiolabelled probe was 0.8-2.0x10^9 cpm/μg DNA.

RNA isolation

About 1.0g tissues were homogenized with 9ml of 4M guanidine isothiocyanate (GIT) in Polytron on ice, then the guanidine lysed samples were laid on the top of 5ml of 5.7mol cesium chrolide buffer and spun for 20 hours at 15°C, 27000rpm. The ob-

tained total RNA samples were quantified by UV absorption at 260nm.

Northern blot hybridization

5.0μg of either total RNA were denatured in resin treated glyoxal mixture at 65°C for 1 hour, and electrophoresed on 1% agarose gel in 10mmol sodium phosphate buffer (pH 7.0), then transferred to Hybond nitrocellulose filter (Amersham, Japan). Prehybridization was performed in 0.5ml/cm^2 of a solution containing 2xSSC (1xSSC = 0.15M sodium chloride, 0.015M sodium citrate), 50mmol Tris (pH 7.4), 1 x Modified Denhardt, 1mol NaCl, 10mmol EDTA, 0.1% SDS and 10μg/ml denatured salmon sperm DNA. Prehybridization was done at 50°C for 1 hour. Hybridization was done in the same buffer (0.1ml/cm^2) plus 10^{6}cpm/ml of radioactive probe and incubated at 65°C for 24 hours. The filters were then washed in 4xSSC, 0.1%SDS for 10 minutes at room temperature followed by 2xSSC, 0.1%SDS at 50°C for 10 minutes, and 0.1xSSC, 0.1%SDS at 61°C for 30 minutes. Filters were then blotted dry and exposed for 24 hours using intensifying screen (Fuji Film Co.,Japan).

Immunohistochemistry

Formalin-fixed tissues were dehydrated and embedded in paraffin. Sections were cut at 4μm, deparaffinized and rehydrated. Immunostaining, using anti-hCRH antibody as first antiserum, was carried out by the avidin-biotin complex method[7], using Vectastin ABC kits (Vector Laboratories Inc., Burlingame, CA). CRH antiserum was raised in rabbits as described in detail elsewhere[8]. Diamino-benzidin tetrahydrochloride was used as peroxidase substrate. The sections were treated with 0.3%H_2O_2 in methanol and 3% normal rabbit serum to reduce nonspecific background staining and block endogeneous peroxidase activity. In addition to the immune serum diluted 1:100, normal rabbit serum was used for controls. In order to allow a reliable histological study of the tissues, hematoxilin-eosin stain was also carried out in each tissue.

Results

Northern blotting

Fig. 1 shows the northern blot analysis of rat hypothalamic and human 40 weeks placental RNA. A single band was hybridized with the probe in rat hypothalamic RNA lane (lane 4) as positive control and in the placental RNA lane (lane 3);both sizes were about 1300 nucleotides, as previously described[6]. No hybridized material was detected in molar RNA lane (lane 1), 10 weeks placental RNA lane (lane 2) and rat liver RNA lane (lane 5) as negative control. Northern blot hybridization analysis of RNA from developing placentae of various stages is shown in Fig. 2; pregnancy 6 weeks (lane 1), 10 weeks (lane 2), 14 weeks (lane 3), 15 weeks (lane 4), 17

weeks (lane 5), 23 weeks (lane 6), 28 weeks (lane 7) and 40 weeks (lane 8). No positive band was detected except in 28 and 40 weeks placental RNA lane. The analysis of placental, amniotic membraneous and uterine myometrial RNA of term pregnancy is also shown in Fig. 3. Lane 1 shows placenta, lanes 2 and 3 show amniotic membranes and uterine myometruim. Single positive bands about 1.3kb were detected in amniotic membraneous and placental RNA lane of 40 weeks of pregnancy, but not in uterine myometrial lane.

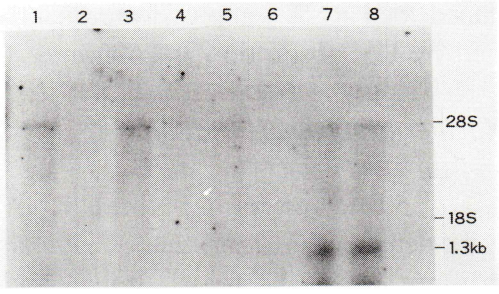

Fig. 2 Northern blot hybridization analysis of RNA from developing placentae. Lane 1, human placenta of 6w pregnancy; 2, 10w; 3, 14w; 4, 15w; 5, 17w; 6, 23w; 7, 28w; 8, 40w. As size markers, 28S and 18S ribosome RNA were shown in right side.

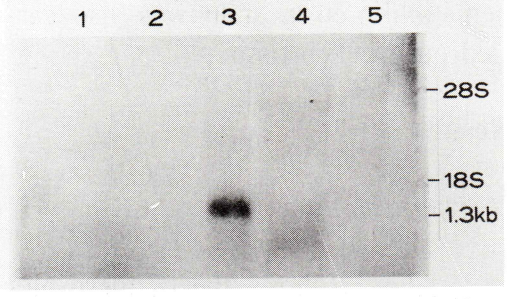

Fig. 1 Northern blot hybridization analysis of rat hypothalamic, liver, human placental and molar RNA. Lane 1, human hydatidiform mole; 2, human placenta (10w); 3, human placenta (40w); 4, rat hypothalamus (positive control); 5, rat liver (negative control). 28S and 18S ribosome RNA were used as size markers, and internal standards (right side).

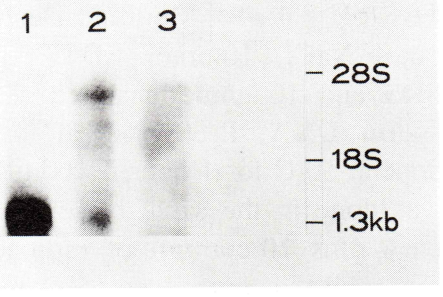

Fig. 3 Northern blot hybridization analysis of placenta, amniotic membranes and uterine myometrium. In right side, 28S and 18S rRNA were shown as size markers.

Immunohistochemistry

In the placenta of 6 weeks of pregnancy, the cytotrophoblast layer beneath the syncytiotrophoblast was evenly stained as shown in Fig. 4. The glandular epithelium was stained in the decidua, and the stromal cells were diffusely positive as well (Fig.5). In term placenta, the staining reaction was negative (Fig.6), but the amniotic membranes were heavily stained (Fig.7). In the tissue of hydatidiform mole, no evidence of immunostain was seen (Fig.8).

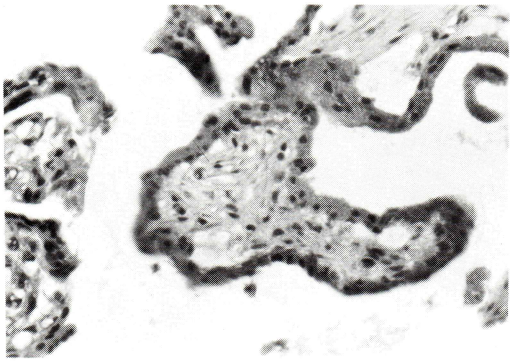

Fig. 4 Immunohistochemical analysis of normal placenta, six weeks of gestation.
(A) Haematoxylin-eosin staining,
(B) control staining,
(C) staining with anti-CRH immune serum. In (C), note that positive reaction is confined to the cytotrophoblast. Original magnification ; x200.

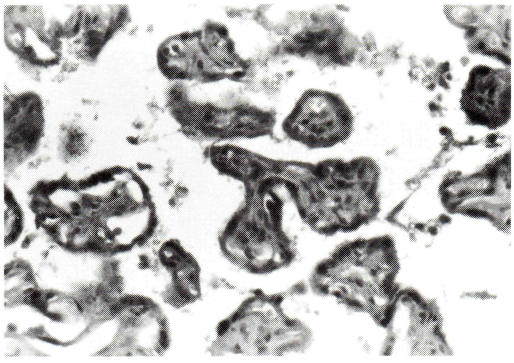

Fig. 5 Normal decidua, six weeks of gestation. Staining is as in Figure 4. Note the positive reaction both in the glandular epithelium and in many stromal cells. Original magnification ; x200.

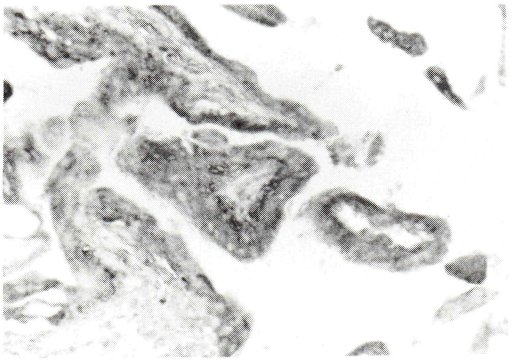

Fig. 4 (B)

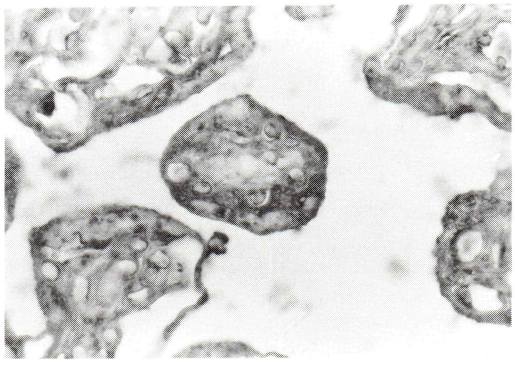

Fig. 5 (B)

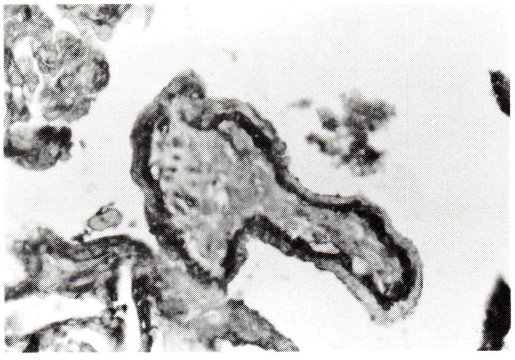

Fig. 4 (C)

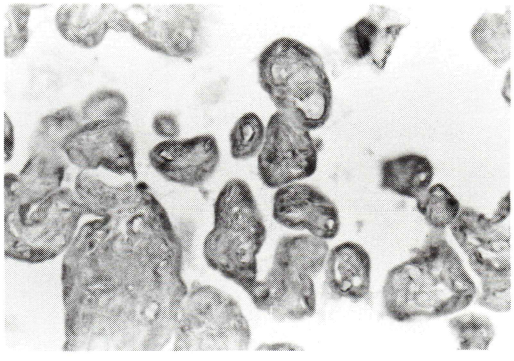

Fig. 5 (C)

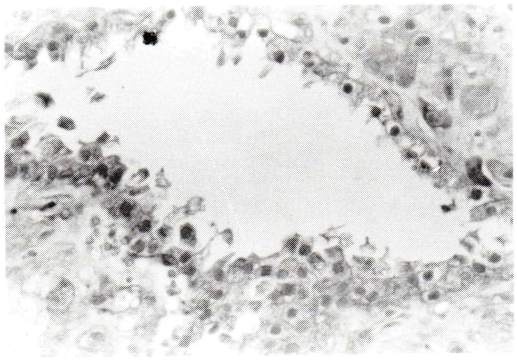

Fig. 6 Normal placenta, 40 weeks of gestation. Note that there is no staining reaction in frame C, where the immune serum has been used. Original magnification ; x200

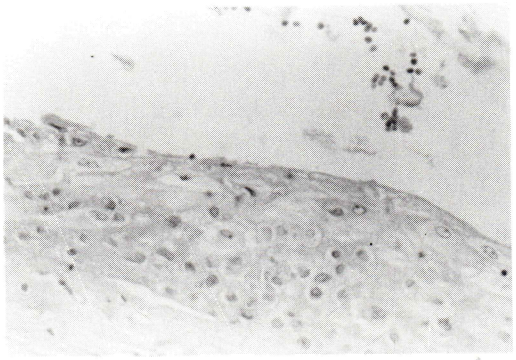

Fig. 7 Normal amniotic membranes, 40 weeks of gestation. Note the strong CRH reaction in the epithelium in frame C. Original magnification ; x200

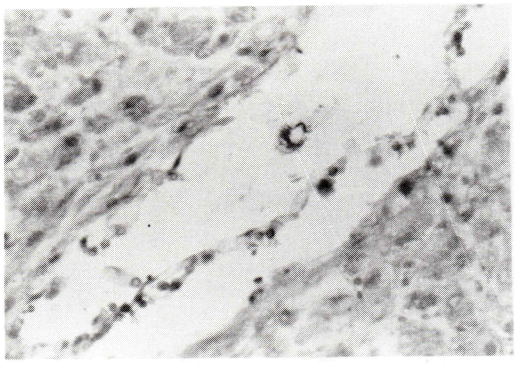

Fig. 6 (B)

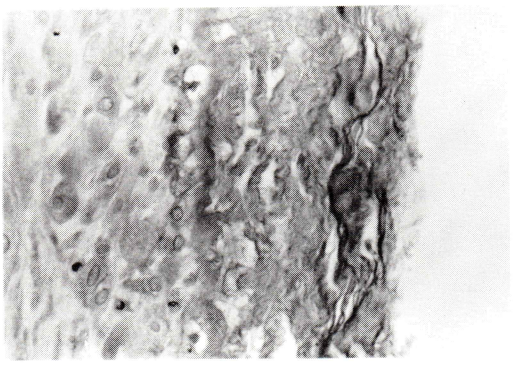

Fig. 7 (B)

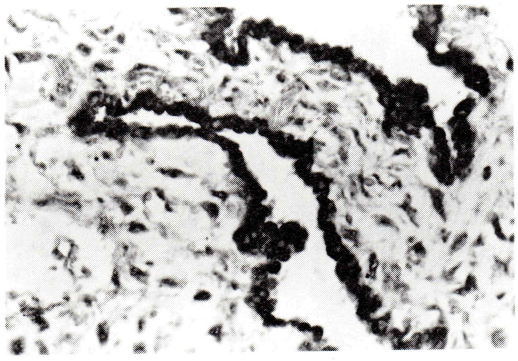

Fig. 6 (C)

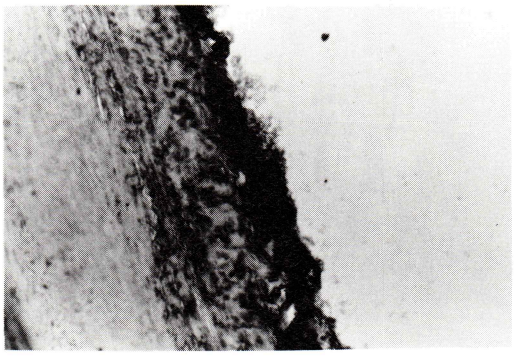

Fig. 7 (C)

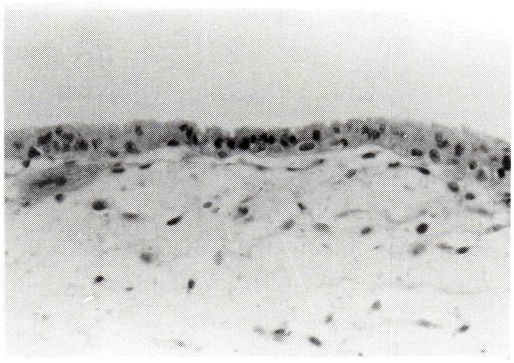

Fig. 8 Hydatidiform molar tissue, 10 weeks of gestation. Note that there is no staining reaction in frame C where anti-CRH serum has been used.

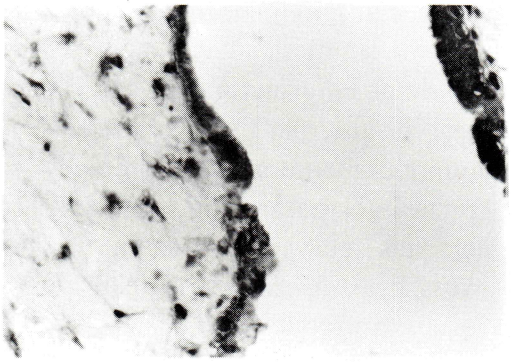

Fig. 8 (B)

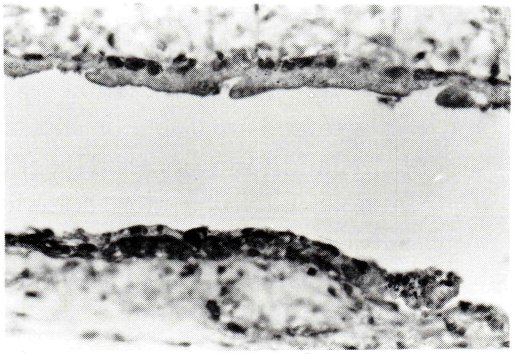

Fig. 8 (C)

Discussion

Although term placenta has a high concentration of IR-CRH (2-25μg/placenta)[9], there has been no systemic study for the expression of hCRH in placenta at various stages of pregnancy, nor in amniotic membranes, uterine myometrium or trophoblastic disease. In this report, we show that the expressions of hCRH gene in placenta do not appear clearly at 6, 10, 14, 15, 17 and 23 weeks of pregnancy, but become evident after 28 weeks of gestation. Thus, it is supposed that the expression of CRH mRNA increases coincidental with the growth and maturation of the placenta. At term pregnancy, the expression of CRH gene in amniotic membranes, which is weaker than that in placenta, is also identified, but not in myometrium which is attached closely to the placenta.

IR-CRH localization was also demonstrated in the cytotrophoblast of placenta and decidua of early pregnancy, and in the amniotic membranes of term pregnancy. The observations that IR-CRH is localized in cytotrophoblast, but that CRH mRNA is below the limit of detection in first and second trimester placentae, support the notion that the localization of IR-CRH may be due to the storage of mitogen, not continuous gene expression. Similar phenomenon has also been observed on fibroblast growth factor in rat ovary[10]. It may be speculated that CRH is stored rather than secreted during early pregnancy, and the ex-

pression of CRH increases as pregnancy advances. At term pregnancy, the rate of CRH secretion exceeds storage and, thus, the proportion of stored CRH may be decreased in addition to the small proportion of cytotrophoblast. Consequently, the immunohistochemical finding of term placenta is negative, which is in accordance with previous reports[11,12].

On the basis of these results, it is sugested that the placenta and amniotic membranes of term pregnancy produce and secrete hCRH, and the production site of increased plasma CRH of pregnant women are these tissues. On the other hand, Laatikainen et al[13] reported that IR-CRH exists in amniotic fluid and it increases greatly during the latter half of pregnancy. It was shown that this increased IR-CRH in amniotic fluid is produced and secreted from amniotic membranes from our results together with their results.

A physiological role for the CRH during pregnancy has yet to be ascertained. It has been reported that CRH binding protein (CRH-BP) exists in human plasma and most of plasma CRH is bound to CRH-BP and inactivated[14,15]. Although a large amount of IR-CRH, most of which is produced from placenta, may be preferentially secreted into the maternal circulation, the influence of maternal pituitary-adrenal axis may be within the physiological range because a large proportion is bound to CRH-BP and inactivated. Another report suggested that CRH may be one of the initiators of labour, because the level of plasma IR-CRH of pregnant women who subsequently went into premature labour was raised several weeks before the onset of labour[16]. The increased IR-CRH and cortisol levels in amniotic fluid are accompanied with raised lecithin/sphingomyelin ratio and phosphatidylglycerol[13]. Similar observations have been reported on prolactin (PRL). IR-PRL in maternal plasma, as well as amniotic fluid increases during pregnancy, and human decidua contains high levels of IR-PRL[17]. It has also been suggested that PRL produced by the decidua is secreted through the fetal membranes into the amniotic cavity and accelerates fetal lung maturation. In view of these results, the CRH produced from amniotic membranes is probably secreted into the amniotic fluid and accelerates fetal maturation in obstetric stress by stimulating the fetal adrenal cortex to produce corticosteroids.

It is reported that the maternal plasma IR-CRH level is in normal non-pregnant range in hydatidiform mole[18]. However, there has been no molecular biological and immunohistochemical study of CRH in molar tissue. Our study shows that CRH m-RNA and IR-CRH localization were not demonstrated in trophoblastic tissue of hydatidiform mole. Hydatidiform mole, which is a most common trophoblastic tumour, has a potential DNA synthesis and rapid growth and proliferation. In this tumour, CRH gene expression was probably suppressed due to the characteristic

change of trophoblast.

In conclusion, CRH is proved to be produced and stored in the placenta during normal pregnancy by both molecular biological and immunohistochemical techniques. Its production/storage ratio changes as pregnancy advances, smaller at early pregnancy and larger at term pregnancy.

Acknowledgement

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