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PHYSIOLOGICAL ROLES OF PLACENTAL PROTEASES IN FETO-PLACENTAL HOMEOSTASIS

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ABSTRACT

Although many proteases exist in human placenta, their physiological roles are still largely unknown. Our studies showed that these placental proteases metabolize vasoactive and immunomodulating peptides, possibly derived from the fetus, and control the exchange of peptide hormones across the placenta in order to maintain feto-placental homeostasis. We clarified the pregnancy serum oxytocinase discovered by Fekete in 1930 and angiotensinase by Page in 1947, respectively. In addition we showed bradykininase in the pregnancy serum. The ratio of peak systolic over least diastolic flow velocity of uterine or umbilical artery assessed by the Doppler technique was closely correlated with the levels of maternal serum proteases in pre-eclampsia, which suggested that placental proteases might control uteroplacental circulation via the regulation of concentrations of vasoactive peptides in uteroplacental circulation. Thus, changes in maternal serum protease activities were useful for monitoring of pre-elcampsia and predicting the onset of labor. The degradation of immunomodulating peptides by placental protease(s) also suggests the possible involvement of placental protease(s) in the immunological aspect of pregnancy. Recently we have cloned one of the major placental protease, oxytocinase (P-LAP). Its amino acid sequences had 87% homology of rat IRAP so called VP 165. This enzyme was confirmed to co-localize in Glut 4 containing vesicles in rat skeletal muscles and adipocytes. Accordingly PLAP, the human homologue of VP165, may have an intriguing possibility in a variety of events not restricted to the regulation of pregnancy induced phenomena.

Key Words: Placental protease, Placental function, Pre-eclampsia, Onset of labor, immunology of pregnancy, PLAP

Recent advances in fetal endocrinology suggest that peptide hormones derived from the fetus are as important in the physiology and pathology of human pregnancy as those from the mother. Human fetuses produce peptide hormones, some of which are considered to be important factors in fetal development and growth,[1-4] the exchange of some peptide hormones between the mother and fetus,however, should be restricted in order to maintain feto-maternal homeostasis. The inability of peptide hormones to cross the placenta contributes partially to such restrictions.

Although many proteases exist in human placenta, their physiological roles are still unknown. It is reasonable to assume that fetal peptides are candidates for natural substrates of placental proteases. This paper reviews the research concerned with placental proteases and their involvement in the metabolism and degradation of peptide hormones in relation to the physiology and pathology of human pregnancy. In addition, we have recently confirmed one of those proteases, oxytocinase, P-LAP, exists in other tissues (i.e. epithelial cells, gastrointestinal mucosal cells, etc.).[5] It is suggested that important roles of these proteases are probable contributing in normal human physiology.

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I. PLACENTAL PROTEASES AND THEIR INVOLVEMENT IN THE METABOLISM AND DEGRADATION OF PEPTIDE HORMONES

Many proteases exist in human placenta and some are released into maternal circulation. A protease is classified according to the peptide site of hydrolysis: exopeptidase consists of aminopeptidases, which hydrolyze from the N-terminal amino acid, and carboxypeptidases, which hydrolyze from the C-terminal amino acid. Endopeptidases hydrolyze the internal peptide bond. The following 9 proteases have been shown to be present in human placenta.

A. Oxytocinase (EC 3.4.11.3)

The oxytocinase that increases in pregnancy serum is identical to cystine aminopeptidase i.e. placental leucine aminopeptidase (P-LAP).[6] The origin of P-LAP was suggested to be placental lysosomes and we purified this enzyme from retro-placental serum.[7] This enzyme hydrolyzes oxytocin, vasopressin and angiotensin III (A-III).[6] It is noteworthy that vasopressin is hydrolyzed at least 3 times faster than oxytocin when monitored by the release of Tyr residue located at the second position from the N-terminal end of both hormones.[6]

B. Aminopeptidase N (EC 3.4.11.2)

Aminopeptidase N (AP-N) is a glycoprotein localized in the microvillar membrane of a variety of human organs, tissues and hematopoietic cells. We purified this enzyme from a human placental microsomal fraction.[7] We used immobilized bestatin, the product of streptomyces and a powerful inhibitor of aminopeptidases N and B, for the affinity column in the purification of this enzyme.[8] Molecular cloning showed that AP-N is identical to the cell surface cluster of the differentiation antigen (CD13).[9]

The function of this enzyme has been uncertain, but recently it was revealed that AP-N hydrolyze the N-terminal arginine of angiotensin III to generate angiotensin IV in vivo.[10] This indicates that AP-N may be an important component of local renin-angiotensin system including placenta.

C. Post-proline endopeptidase (EC 3.4.21.26)

Walter *et al.* demonstrated the existence of an enzyme that cleaved the Pro7-Leu8 peptide bond of oxytocin in the human uterus, designated it as post-proline endopeptidase and purified from lamb kidney.[11] They showed that the enzyme responsible for oxytocin degradation was the cytosolic post-proline endopeptidase. We purified this enzyme from human placenta[12] and tried to confirm the degradation of oxytocin to no avail. The human placental post-proline endopeptidase did not liberate any amino acids from oxytocin,[13] but on the other hand, it liberated the C-terminal Phe from angiotensin II (A-II). Recently Tan *et al.* cloned lysosomal carboxypeptidase (EC 3. 4. 16. 2) (angiotensinase C) and found some sequence similarity to human post-proline endopeptidase.[14]

D. Angiotensin-converting enzyme (ACE, kininase II, EC 3.4.15.1)

This enzyme converts angiotensin I (A-I) to A-II by cleaving the C-terminal dipeptide, His-Leu, of A-I and also inactivates bradykinin (BK) by cleaving the C-terminal and adjacent peptides. We purified this enzyme from human placenta by affinity chromatography with captopril, an orally active antihypertensive agent and potent inhibitor of this enzyme.[15] Soubrier *et al.* cloned this enzyme.[16] We showed that two different sizes of ACE mRNA, 3.9kb and 4.5kb, are detectable in human placenta.[17]

E. Aminopeptidase A (AP-A, EC 3.4.11.7)

This enzyme hydrolyzes N-terminal acidic amino acids, such as aspartic and glutamic acid, from the peptide or synthetic substrate. We purified this enzyme from human placenta by the immnoaffinity-chromatography using antibody against AP-N.[18,19] We showed that AP-A metabolizes and converts A-II to A-III.[20] Molecular cloning showed that AP-A is identical to the cell surface glycoprotein (gp160), BP-1 molecules immnoprecipitated from muriro pre-B cells.[21]

F. Carboxypeptidase N (kininase I, EC 3.4.17.3)

This enzyme hydrolyzes the C-terminal basic amino acids of peptide substrates and that is an inactivator of peptides such as BK, anaphylatoxin and fibrinopeptides. We purified this enzyme from human placenta using affinity chromatography with histargin, a potent inhibitor of this enzyme discovered by Aoyagi *et al.* from the products of streptmyces.[22] Molecular cloning of this enzyme, purified from membrane fractions of various organs and human plasma, showed the molecular difference between membrane (carboxypeptidase M) and serum (carboxypeptidase N) enzymes.[23]

G. Dipeptidyl peptidase IV (EC 3.4.14.5)

This enzyme is localized on the cell surface and cleaves N-terminal dipeptides from polypeptides containing Pro as the penultimate amino acids, such as substance P, fibrin a-chain and casmorphine 5. We purified this enzyme from human placenta by affinity chromatography with the dipeptide Gly-Pro.[24] The primary structure of this enzyme was deduced from its cDNA and the human CD26 antigen was identified as this enzyme.[25] This enzyme was also shown to be involved in the interaction of hepatocytes with the extracellular matrix.[26]

H. Aminopeptidase B (AP-B, EC 3.4.11.6)

This enzyme liberates N-terminal basic amino acids such as Arg and Lys from synthetic substrates or peptides. It is widely distributed in mammalian tissues and is a sulfhydryl-dependent cytosolic enzyme. We purified this enzyme from human placenta.[27] Although its involvement in the formation of BK from kallidin-l0 was suggested, this enzyme does not seem to be involved in the metabolism of kinins and A-III.[27] The natural substrate of this enzyme is still unknown.

I. Aminopeptidase P (AP-P, EC 3.4.11.9)

This enzyme hydrolyzes N-terminal amino acid residues linked to Pro in peptides of low and high molecular weight. It is probable that, together with ACE and kininase I, AP-P is responsible for the inactivation of circulating BK.[28] Hooper and Turner showed that AP-P is the ectoenzyme anchored to cell membrane via glycosyl-phosphatidyl-inositol (PI).[29] We found this enzyme in human placenta and also found the enzymatic difference between cytosolic and membrane AP-P. AP-P in the placental microvillar membranes was released by PI-specific phospholipase C, and pregnancy serum AP-P was considered to be derived from placental microvillar membranes.[30]

We found significant increases in P-LAP,[31] AP-A,[32] kininase I,[33] AP-P[34] and ACE (kininase II)[35] in maternal serum during normal pregnancy.

II. REGULATION OF BIOSYNTHESIS AND SECRETION OF PLACENTAL PROTEASES

During pregnancy, steroid hormones such as progesterone, estradiol, and cortisol are synthesized in the feto-placental unit. The production of these steroids in the feto-placental unit and their concentrations in the maternal and fetal circulation increase with advancing gestation, but little is known about specific functions of the steroids on the development and metabolic well-being of the fetus. On the other hand, a possible site of production of pregnancy serum oxytocinase (P-LAP) is shown to be the lysosomes of the placenta,[7] from which the enzyme is released into the maternal circulation presumably through changes in membrane permeability. In view of the fact that progesterone acts to destabilize the membrane of lysosomes, while cortisol acts to stabilize them, steroid hormones might play a part in such changes in the permeability of the lysosomal membranes to oxytocinase and thus regulate its levels in pregnancy serum.[36]

We investigated the effect of estradiol plus progesterone on placental proteases in pregnant rats. The administration of both steroids increased the levels of LAP in serum (oxytocinase) and AP-A in serum and membrane fractions of placenta.[37] We also showed that cortisol increased the activity of AP-N, AP-A and post-proline endopeptidase by using short-cultured human placental tissues.[38] It should be noted here that it is known that metalloendopeptidase is inducible by glucocorticoids.

Several researchers have reported that the administration of oxytocin during pregnancy resulted in an increase in oxytocinase in the maternal plasma.[39] Therefore, it is reasonable to presume that the proteases in serum during pregnancy are under the influence of feto-placental steroids and/or peptide hormones.

III. PHYSIOLOGICAL SIGNIFICANCE OF PLACENTAL PROTEASES

A. Mechanisms of onset of labor and transient diabetes insipidus

The pregnant uterus is very sensitive to exogenously infused oxytocin at term compared with that in the 2nd and 3rd trimesters. Oxytocin is therefore widely used for initiating human labor. The physiological importance of oxytocin for uterine activity in pregnant women has gained much attention following the cloning of the oxytocin receptor[40] and the demonstration of an increase in the number of receptors in term uterus.[41,42] In addition, the development of specific oxytocin inhibitors with therapeutic effects on preterm labor has further stimulated interest in this field.[43]

On the other hand, there was a significant increase in maternal plasma oxytocin with advancing gestation, although pronounced fluctuations in its levels in pregnant women are known.[44] Therefore, the maintenance of gestation despite the increased presence of oxytocin receptors in the uterus, is strange. In relation to the role of the oxytocin-oxytocinase system in forwarding the onset of labor, the changes in P-LAP (oxytocinase) activities during late pregnancy are suggestive of the role and useful for predicting the onset of labor. The mean serum P-LAP levels during the last 28 days of pregnancy showed that a maximum level was reached at 11 days before the onset of labor and did not increase thereafter.[44] Labor may be initiated by an increase in oxytocin release together with a decrease in oxytocin degradation by oxytocinase. It is clear that parturition is a highly regulated phenomenon and likely involves the interplay of many additional factors, but the most reasonable assumption concerning the function of oxytocinase is that it serves to regulate the oxytocin level of maternal plasma and thus prevents premature labor.

The metabolic clearance rate of vasopressin, possibly due to an increase in P-LAP activity during the 2nd and 3rd trimesters, was much higher than in the 1st trimester as well as in non-pregnant women. The increase in the vasopressin disposal rate may explain the appearance of transient vasopressin-resistant diabetes insipidus during pregnancy.[45]

B. Effects of placental proteases on fetal blood pressure

The administration of AP-N and AP-A purified from human placenta was effective in lowering blood pressure in rats with experimental hypertension induced by the infusion of A-II or renin.[46] Placental proteases had an effect on blood pressure via the degradation of A-II *in vivo*. In addition, the administration of AP-A and AP-N was also effective in lowering the blood pressure of spontaneously hypertensive rats (SHR).[47] Since ACE inhibitor normalizes the blood pressure of SHR, the renin-angiotensin system may be involved in the high blood pressure of SHR.[48] Therefore the hypotensive effects induced by these two enzymes are likely to be due to A-II degradation by both enzymes.

Although maternal cardiac output increases 40% and circulatory blood volume increases 50% in the late stage of normal human pregnancy, blood pressure remains stable. The circulation of the fetus is also characterized by high cardiac output and blood volume (per kilogram weight) relative to that of the adult, but the mean fetal capillary pressure is 20–40 mmHg. Since blood pressure is a function of cardiac output and peripheral vascular resistance, the decrease in vascular resistance in the whole body, especially in the uteroplacental circulation, is essential for the maintenance of normal blood pressure during pregnancy.

Uteroplacental circulation consists of the intervillous (retroplacental blood pool) and villous systems. In regard to the intervillous system, the coilled artery, which branches off uterine artery and is found in the decidual part of the placental bed, supplies blood to the retroplacental pool, reaching a flow of 400–500 ml/min near term. It is known that the normal musculoelastic tissue in the vessel wall of the coilled artery is replaced by fibrinous tissue with advancing gestation. Therefore the vascular resistance of the coilled artery is controlled only by uterine contraction which in turn effects the volume of the retroplacental pool.

The villous system, which is supplied with blood from the umbilical blood flow which can reach 350–400 ml/min at term, is best compared with an inverted tree.[49] The regulation of blood pressure is mainly influenced by autoregulation, the autonomic nervous system and the humoral system, but the placenta (uteroplacental circulation) is not capable of autoregulation;[50] it appears to be totally dependent on perfusion pressure. Although the autonomic nervous system in the fetus develops with advancing gestation, fetal end organ response to the autonomic nervous system (in both blood vessels and the heart) is of a much smaller magnitude than the adult response.[51,52] Blood pressure homeostasis may be more important in the fetus than in the adult, as it determines the level of placental perfusion which then determines the extent of respiratory gas exchange.

The human fetus produces equal or higher levels of various vasoactive peptides, such as A-II, vasopression and somatostatin compared with the mother.[2,4,53,54] It appears that A-II plays an important role in normal cardiovascular physiology and could play a role in aberrent conditions. Vasopression may be one cause of the rise in blood pressure, fall in heart rate, and redistribution of blood flow that occurs during fetal distress.[55] The use of ACE inhibitors in human pregnancy is known to cause intrauterine growth retardation (IUGR) and severe disturbance of fetal and neonatal renal dysfunction due to severe drug-induced fetal hypotension: this strongly suggests the involvement of placental proteases, such as ACE and their substrates such as A-I and BK and/or products thereof, in the maintenance of uteroplacental circulation.[56] With regard to the mechanism for the maintenance of uteroplacental circulation, it is reasonable to speculate that

the vascular resistance of the intervillous and villous systems is at least partly controlled by vasoactive peptides; the concentration of peptides controlled by the production in the fetal site and the degradation by placental proteases may directly affect the uteroplacental circulation.

IV. INVOLVEMENT OF PLACENTAL PROTEASES IN PRE-ECLAMPSIA

Although human and animal pregnancy is normally associated with a refractory response to the pressor effect of exogenously infused A-II, patients with pre-eclampsia as well as nonpregnant women are sensitive to the pressor effect of A-II; this phenomenon has been studied as one of the potential causes of pre-eclampsia.[57] In contrast to A-II we found that there existed a hypersensitivity to the depressor action of BK in pregnant rats.[58] There is evidence that placental proteases effect the altered response of pregnant women to exogenously infused peptide hormones via their degradation. Actually we have shown that the decreased pressor responsiveness to A-II in pregnancy is caused partly by increased inactivation of A-II by AP-A and AP-N in rats.[59]

Higher levels in the fetus of A-II at pre-eclampsia,[2] and vasopressin at hypoxemia[4] than in normal pregnancy may result in changes in uteroplacental resistance. The passage of vasoactive peptides from the fetus to maternal sites may result in the reduction of the retroplacental pool due to uterine contraction. We found interesting patterns of changes in the serum P-LAP of patients with pre-eclampsia. Serial measurements of serum P-LAP activity in patients with severe pre-eclampsia showed a maximum level at week 31, where the values were significantly higher than in normal pregnancy, and at that stage patients were still mildly pre-eclamptic. After week 35, the activity decreased sharply toward week 40. The patients were severely pre-eclamptic during weeks 39 and 40 and the enzyme activity was much weaker than in normal pregnancy.[60]

Since the level of maternal serum P-LAP and AP-A might reflect the production rate in the placenta, the decreased level of serum proteases in severe pre-eclampsia might result in the decrease in the breakdown of hypertensive peptides such as A-II and vasopressin.

On the other hand, we also found another interesting pattern of changes in kininase I in patients with pre-eclampsia. The kininase I activity in severe pre-eclampsia was higher than that in normal pregnancy.[33] Since maternal serum kininase I level might reflect the production rate in the placenta, the increased levels of serum kininase I in severe pre-eclampsia might result in hte increase in the breakdown of hypotensive peptide, BK.

It would be interesting to know whether changes in maternal serum proteases, which degrade vasoactive peptides are reflected in the changes in the concentrations of those peptides in the uteroplacental circulation. The Doppler technique provides a good index of placental blood flow resistance. Campbell *et al.* showed that patients with pre-eclampsia were associated with an increase in the ratio of peak systolic over least diastolic (S/D) flow velocity measured in the uterine or umbilical artery.[61] We found significant correlations between the S/D values and the levels of maternal serum proteases in pre-eclampsia. While kininase I was positively correlated with the S/D values, P-LAP and AP-A were negatively correlated with the S/D values. These correlations in pre-eclampsia might therefore indicate the changes in vascular resistance in the uteroplacental circulation via changes in the vasoactive peptides concentrations due to degradation by placental proteases.[62]

V. POSSIBLE ROLE OF PLACENTAL PROTEASES IN IMMUNE SYSTEM DURING PREGNANCY AND EFFECT ON FETAL GROWTH

Accumulating evidence supports the hypothesis that cellular proteases play an important role in the communication among cells in the immune system.[63] It has been suggested that many immunomodulator peptides present in thymic and splenic extracts, such as thymopoietin,[64] splenin[65] and tuftsin[66] influence the immune system. Recently a possible link between the endocrine and immune systems has been shown: these include derivatives of pro-opiomelanocortin such as Met-enkephalin,[67] and also somatostatin[68] and tachykinin,[69] which are known to be present in umbilical blood.[53] As previously mentioned, we found that AP-N hydrolyzes these peptides and various immunomodulating peptides *in vitro*. Therefore not only placental membrane proteases identified as CD, such as AP-N (CD13), depeptidyl peptidase IV (CD26)[70] and endopeptidase 24. 11 (CD10),[71] but also AP-A and AP-P in placental membranes may be involved in the immune system during pregnancy by regulating the action of immunomodulating peptides.

Previously we found that the administration of bestatin, a specific inhibitor of AP-N and AP-B, to pregnant rats resulted in growth-retarded fetuses: placental protease might play an important role in fetal growth[72] via regulation bioactive peptides. It is known that almost no bestatin is transfered to rat fetuses through the placenta.

Although about a quarter of a century ago we initiated our research in order to provide a services of a placental function tests, at present the research field of placental proteases may involve not only the pathophysiology of pregnancy i.e. onset of labor and pre-eclampsia, but also the immunology of pregnancy.

VI. FURTHER EVOLUTION OF PLACENTAL PROTEASE; ROLE OF OXITCINASE (P-LAP) IN MAINTAINANCE OF HUMAN HOMEOSTASIS

Recently, we confirmed wide distribution of P-LAP not only in placenta and fetal tissues but also in adult tissues by RNA analysis in addition to immunohistochemistry.[5] In both fetal and adult tissues, positive staining was obtained in vascular endothelial cells, gastointestinal mucosal cells, epithelial cells of hepato-biliary, pancreato-biliary, bronchial-alveolar and renal tubular systems as well as islet cells of pancreas and neurons in the central nervous systems. Sweat-gland cells, seminal vesicles and prostate gland in the adult, as well as adipocytes and skeletal muscle cells in the fetus were also stained. The wide spread distribution of P-LAP suggests its involvement in a variety of physiological events not restricted to the regulation of the amounts of bioactive peptides such as arginine vasopressin (AVP) and oxytocin in pregnancy.

We have purified P-LAP from human retroplacental sera and cloned its cDNA from a human placental cDNA library. Recently, from a rat adipocyte cDNA library, a cDNA has been cloned that encodes a novel insulin-regulated membrane aminopeptidase (IRAP) present in vesicles carrying glucose-transporter 4 (GLUT4).[73] Its amino acid sequence and that of P-LAP can be aligned closely without introducing any deletion or addition except at the C-terminals. Their high degree of similarity is indicated by 87% of their amino acid sequences being identical. Thus this enzyme is almost certainly the human homologue of IRAP.

The possible physiological significance of P-LAP suggested by its wide tissue distribution in not only pregnant but also in non-pregnant women and men is truly intriguing. It is likely that the role of this enzyme is not limited to the degradation of bioactive peptides, and the possibility

of a wider role warrants further extensive studies.

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