

Expression of Antiangiogenic Prolactin Fragments in the Placentas of Women with Pregnancy Induced Hypertension

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It has been reported that prolactin (PRL) is cleaved to 14 or 16 kDa fragments by cathepsin D *in vitro* and *in vivo*, and that such fragments exhibit antiangiogenic and proapoptotic properties. The aim of this study was to investigate the relationship between pregnancy induced hypertension (PIH) and the placental expression of antiangiogenic PRL fragments and cathepsin D. Placental expression of PRL fragments and cathepsin D was evaluated by Western blot analysis in a group of 9 pregnant women consisting of 5 normal pregnancies and 4 severe PIH cases. Antiangiogenic PRL fragments were detected in 4 placental samples from all PIH cases but not detected in those from normal pregnancies ($p < 0.05$). The expression of cathepsin D in PIH placentas was significantly lower than that in those without PIH ($p < 0.05$), while the placental expression of procathepsin D was significantly greater in PIH cases than in the normal pregnancies ($p < 0.05$). These data suggest that antiangiogenic PRL fragments in the placenta may be present only in PIH cases, and that PRL fragments in the placenta might be implicated in the pathophysiology of PIH.

Key words: pregnancy induced hypertension, preeclampsia, prolactin, prolactin fragment, cathepsin-D

Prolactin (PRL) is synthesized and excreted in the anterior pituitary and has a wide range of physiological effects, including effects on osmosis, immunoregulatory function, luteal function, and sexual behavior, as well as mammary gland development and galactopoiesis during the lactational stage.

This hormone is also produced in many tissues in addition to the anterior pituitary and is known to act locally [1]. It has been reported that the normal 23 kDa PRL has angiogenic action both *in vitro* and *in vivo*, while the 14kDa and 16kDa PRL fragments

cleaved from the 23kDa PRL under a low pH environment with cathepsin D, which is lysosomal protease, have antiangiogenic and proapoptotic properties, respectively [2].

Pregnancy induced hypertension (PIH), or preeclampsia, is a major cause of maternal mortality and morbidities, perinatal deaths, preterm birth, and intrauterine growth restriction, and its mechanism has not been identified thoroughly [3]. PRL increases during pregnancy and the major circulating PRL isoform (23kDa) has angiogenic action while the 16kDa fragments resulting from proteolytic cleavage from 23kDa PRL have antiangiogenic action. Therefore, it has been hypothesized that the balance between the 23kDa PRL and 16kDa PRL plays an important role in the placenta and that if the 16kDa PRL becomes

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predominant over the 23kDa PRL in the early stage of pregnancy, this imbalance induces preeclampsia due to the shallow entry of the spiral artery [4, 5].

It has been reported that the levels of the 14 and 16kDa antiangiogenic PRL fragments are increased in the urine and amnion liquid in preeclampsia cases, and renal glomerulus and deciduas have been suggested as the source of these increases, respectively [6, 7]. It is suspected that the PRL fragments are associated with PIH; however, there has been no report of the increased PRL fragment expression in the placenta of women with PIH.

Cathepsin D is observed as a lysosomal protease in almost all cells, tissues and organs [8] and processes PRL into N-terminal fragments such as 14kDa or 16kDa PRL [2, 9]. Cleavage of PRL by cathepsin D generates N-terminal fragments that act on endothelial cells to suppress vasodilation and angiogenesis and promote vascular regression [10], suggesting that the expression of cathepsin D might play an important role along with antiangiogenic fragments of PRL in the placenta.

The aim of this study was to investigate the relationship between PIH and the placental expression of antiangiogenic prolactin fragments and cathepsin D.

Materials and Methods

Reagents and chemicals. Unless otherwise specified, all reagents and chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Sample collection. The protocol of this study was approved by our institute's review board. All participants were admitted into our hospital, and provided written informed consent. Placental tissue biopsies were obtained during Cesarean section from 9 pregnant women, or 5 normal pregnancies and 4 severe PIH cases. All patients with PIH had severe new-onset hypertension which was defined as systolic blood pressure more than or equal to 160mmHg and/or diastolic blood pressure more than or equal to 110mmHg and an age of 32 weeks or older, while participants assigned to the control group were normotensive. Three of the patients with PIH had massive proteinuria [$\geq 5\text{g/day}$ or $\geq 300\text{mg/dl}$] and the remaining patient did not. Protein extracts from placental tissues were obtained using T-PER tissue pro-

tein extraction reagent (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's protocol and stored at -80°C until analysis.

Western blot analysis. Placental tissue extracts were separated on a 10–20% (for PRL) or a 10% (for cathepsin D) SDS-PAGE gradient gel (System Instruments Co., Ltd., Tokyo, Japan) along with molecular weight markers (Precision Plus Protein Kaleidoscope prestained standards; Bio-Rad Laboratories Inc., Hercules, CA, USA), and subsequently transferred to polyvinylidene fluoride membranes (GE Healthcare UK, Ltd., Buckinghamshire, UK). Then, total protein bands were visualized by Ponceau S (Sigma-Aldrich Co., St. Louis, MO, USA) staining of the membrane to confirm equal protein loading. Nonspecific protein binding was blocked with 8% non-fat dry milk in Tris-buffered saline solution with 0.05% Tween 20 (Sigma-Aldrich Co.) (TTBS) for 2 h at room temperature. The blots were incubated with either rabbit monoclonal antibody raised against PRL (1 : 800 dilution) (the generous gift of Dr. Ingrid Struman, University of California School of Medicine, San Francisco, CA, USA) or β -actin (1 : 1000 dilution) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in TTBS with 3% bovine serum albumin (BSA), or mouse monoclonal antibody raised against cathepsin D (1 : 3000 dilution) (Abcam PLC, Cambridge, UK) or β -actin (1 : 1000 dilution) in TTBS with 3% BSA at 4°C overnight. The blots were washed, then incubated with goat anti-rabbit secondary antibody (1 : 40000 dilution) (KPL Inc., Gaithersburg, MD, USA) for PRL and β -actin or goat anti-mouse secondary antibody (1 : 40000 dilution) (KPL Inc.) for cathepsin D in TTBS for 2 hours at room temperature. The blots were then washed, and specific signals were detected using SuperSignal West Pico chemiluminescent Substrate (Pierce Biotechnology Inc.) according to the manufacturer's instructions. The housekeeping gene β -actin was used to confirm equal loading. The amount of each protein was quantified densitometrically using an Image Scanner CanoScan D 2400U (Canon, Tokyo, Japan) and Bio Image BQ 2.0 software (Bio Image, Ann Arbor, MI, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from placental tissues with or without PIH using Trizol reagent (Life Technology Inc., Grand Island, NY,

USA). Each sample was treated with DNase I to remove genomic DNA contamination. To generate first-strand DNA, 0.1 μ g of total RNA was reverse-transcribed at 42°C for 20 min in 20 μ l of reaction solution containing 1xPCR buffer, 5 mM MgCl₂, 1 mM dNTPs, 2.5 μ M random 9-mer primer, 10 U RNase inhibitor and 5 U AMV reverse transcriptase, using an RNA PCR kit (TAKARA Co., Ltd., Kyoto, Japan) according to the manufacturer's protocol. The primers used to amplify human PRL and GAPDH have been described previously [11]. Amplification of PXR and GAPDH was carried out on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The number of PCR cycles resulting in PCR products in the linear logarithmic phase of the amplification curve was determined. PCR samples were electrophoresed on 3% Nu-Sieve agarose gels and visualized by staining with ethidium bromide. The housekeeping gene glyceraldehydes phosphate dehydrogenase (GAPDH) was used to control for variations in RNA recoveries from each specimen.

Statistical analysis. The data are presented as the mean \pm S.E.M. Statistical analyses were performed using the Mann-Whitney U-test and chi-square test for comparison between each groups. The analyses were performed with the software package StatMate III (Avice Inc., Tokyo, Japan). Differences were considered significant at $p < 0.05$.

Results

Background of patients. The patients consisted of 4 severe PIH cases and 5 control cases. There were no significant differences between PIH and control patients with regard to maternal age, the

number of pregnancies, parity, BMI before pregnancy, gestational week of delivery, or birth weight. Small for gestational age (SGA) infants were observed in 3 out of 4 cases in the PIH group, and the frequency of SGA infants was significantly higher in the PIH group. As expected, systolic and diastolic blood pressures and the frequency of small for gestational age infants were significantly higher in women with PIH (Table 1).

Expression of 23-kDa PRL and 14 or 16-kDa PRL fragments. The level of expression of 23 kDa PRL was greater in the PIH group than in the control group; however, the difference was not significant (Fig. 1A). RT-PCR analysis revealed that the expression of PRL mRNA in the PIH group was also greater than that in the control group (Fig. 1B). On the other hand, the frequencies of the 14 kDa and 16 kDa PRLs were significantly higher in the PIH groups than in the control group, where these PRLs were not observed at all (Fig. 2).

Expression of cathepsin D or procathepsin D. Mature cathepsin D of 34 kDa is produced from 48 kDa cathepsin D of intermediate type processed from 52 kDa procathepsin D [8]. The antibody used in the present study can detect isoforms of 52 kDa, 48 kDa and 34 kDa. The level of mature cathepsin D (34 kDa) was significantly lower in the placentas of the PIH group compared with those of the control group (Fig. 3). In contrast, the intermediate form of cathepsin D (48 kDa) was increased in the PIH group, but not significantly (Fig. 4), and procathepsin D (52 kDa) was significantly increased in the placentas in the PIH group compared with the control group (Fig. 5).

Table 1 Clinical characteristics of pregnant women with severe PIH or normal pregnancies

| Variable | severe PIH (n = 4) | control (n = 5) | P value |
|---|--------------------|-----------------|------------|
| Mean age \pm SEM (yr) | 34.3 \pm 3.73 | 34.2 \pm 0.8 | NS |
| Mean gravidities \pm SEM | 1.75 \pm 1.44 | 0.71 \pm 0.32 | NS |
| Mean parities \pm SEM | 1.5 \pm 1.5 | 1.4 \pm 0.2 | NS |
| Mean body mass index \pm SEM | 27.1 \pm 2.6 | 22.4 \pm 1.9 | NS |
| Mean gestational age at delivery \pm SEM (wk) | 35.0 \pm 0.58 | 32.6 \pm 1.6 | NS |
| Mean infant birth weight \pm SEM (g) | 1,833 \pm 277 | 1,981 \pm 364 | NS |
| No. of SGA infants | 3 | 0 | $p < 0.05$ |
| Mean systolic blood pressure | 179 \pm 4.07 | 111 \pm 3.23 | $p < 0.05$ |
| Mean diastolic blood pressure | 105 \pm 2.4 | 60.8 \pm 3.68 | $p < 0.05$ |

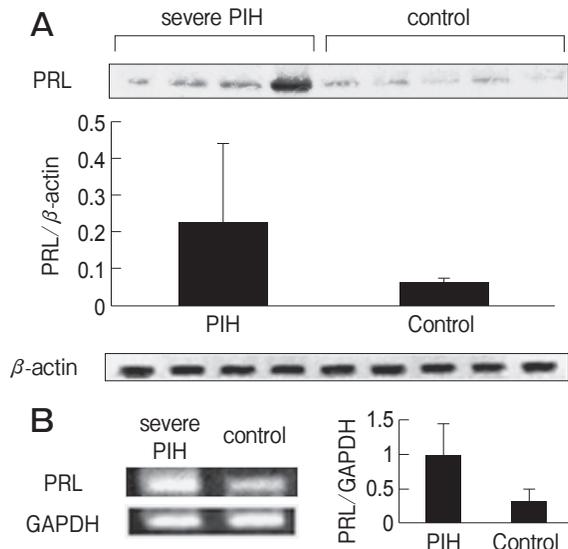


Fig. 1 **A**, Western blot analyses for 23kDa PRL expression in the placental tissues of cases of PIH (n = 4) or normal pregnancy (n = 5). The results represent the mean \pm S.E.M.; **B**, Total RNA was also obtained from the placental tissues of cases of PIH (n = 3) and normal pregnancy (n = 3) and analyzed for the expression of PRL and GAPDH mRNAs using RT-PCR. PCR products were separated on 3% Nu-Sieve agarose gels and visualized by ethidium bromide staining.

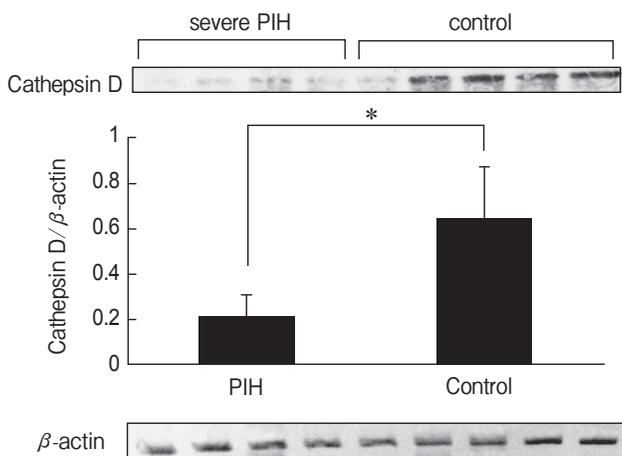


Fig. 3 Western blot analyses for 34kDa cathepsin D expression in the placental tissues of cases of PIH (n = 4) or normal pregnancy (n = 5). The results represent the mean \pm S.E.M. (* p < 0.05).

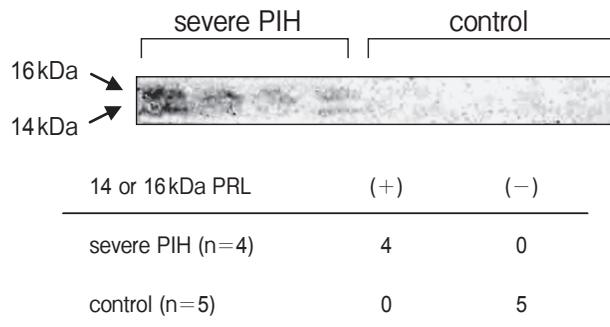


Fig. 2 Western blot analyses for 14 or 16kDa PRL expression in the placental tissues of cases of PIH (n = 4) or normal pregnancy (n = 5).

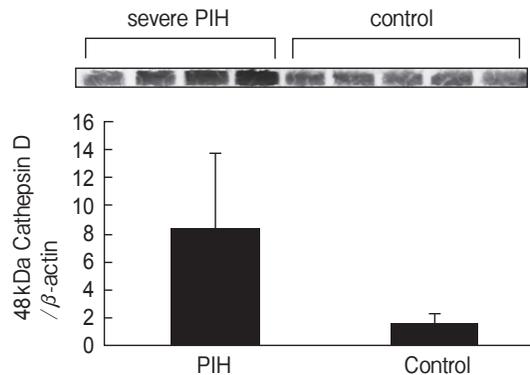


Fig. 4 Western blot analyses for 48kDa cathepsin D expression in the placental tissues of cases of PIH (n = 4) or normal pregnancy (n = 5). The results represent the mean \pm S.E.M..

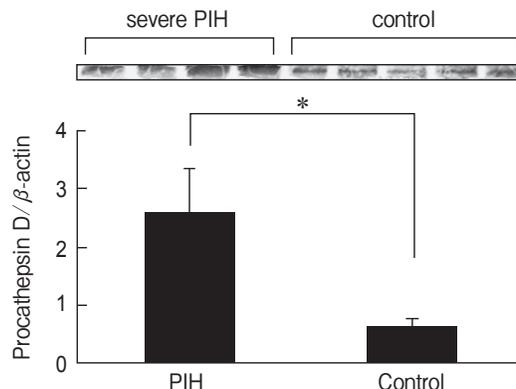


Fig. 5 Western blot analyses for 52kDa procathepsin D expression in the placental tissues of cases of PIH (n = 4) or normal pregnancy (n = 5). The results represent the mean \pm S.E.M. (* p < 0.05).

Discussion

PRL has various isoforms; the 23kDa PRL is the most prevalent and has normal physiological effects [12]. In the present study we observed a higher level of 23kDa PRL expression in the PIH group relative to the control group, but this difference was not statistically significant. However, recent reports have demonstrated that there were no significant differences of 23kDa PRL in blood serums, the urine and amniotic liquid between PIH cases and normal pregnant women [6, 13–15]. PRL fragments that are proteolytically cleaved from 23kDa PRL have been detected in human living bodies as well as *in vitro* and are reported to be associated with various disorders [2]. In the case of pregnancy, it is reported that the fragments are observed in the blood sera of patients with postpartum cardiomyopathy or in the urine or amniotic liquid in preeclampsia cases [6, 7, 16]. Leñanos-Miranda *et al.* reported significant differences of PRL in the urine and that their isoforms appeared to be suitable markers to assess the severity of preeclampsia [7]. To our knowledge, however, there has been no report about PRL and its fragments in the placenta. In this study, we firstly observed that there was no significant difference of 23kDa PRL expression, but the significant increase of antiangiogenic PRL fragments in the placentas of the PIH cases compared with those in the placenta of normal pregnancy.

In this study, PRL fragments of 14kDa and 16kDa were observed in all the placentas in the PIH group, but not in the control group. Isoforms of 14 and/or 16kDa PRL fragments have previously been observed in the pituitary adenoma [9], endothelial cells [17], subretinal fluid [18] and serum [18–21]. Leñanos-Miranda reported that no PRL fragments were observed in the urine of a control group, and that the expression ratio of PRL fragments in severe preeclampsia cases was only 21.6%, which is not a particularly high value [7]. González *et al.* reported that PRL fragments in the amniotic liquid had significantly large amounts of protein in preeclampsia cases. There were some cases in which PRL fragments were observed in the amniotic liquid of cases without preeclampsia [6]. It is highly likely that the expression ratio of PRL fragments varies depending on the tissues even in the PIH cases, however, that antiangio-

genic fragments of PRL might be dominant in the fetoplacental units, including placenta and amniotic liquid, in PIH cases.

We measured the expression of cathepsin D, which processes PRL to antiangiogenic fragments such as 14kDa or 16kDa PRL under acid environments, in the placenta. Mature 34kDa cathepsin D is observed in almost all cells, tissues and organs as a lysosomal protease and is produced from 48kDa cathepsin D of intermediate type processed from 52kDa procathepsin D [8]. In the present study, the level of 52kDa procathepsin D was significantly higher and the level of mature cathepsin D of 34kDa was significantly lower in the patients with PIH than in the controls. Cathepsin D activity has been reported to be increased in the trophoblasts of their PIH subjects [6], while an acidic environment existed in the placenta in patients with preeclampsia and placental hypoxia [22]. The lower level of cathepsin D and higher level of procathepsin D in PIH patients in the present study might indicate that PRL cleavage occurs with cathepsin D in the placenta under an acid environment due to the low oxygen levels and increase in cathepsin D consumption in the placentas of patients with PIH. However, in addition to cathepsin D enzymatic activity, recent reports have demonstrated that procathepsin D acted as a mitogen on both cancer and stromal cells and stimulated their pro-invasive properties, and that the procathepsin D/cathepsin D level represented an independent prognostic factor in a variety of cancers, including endometrial cancers and ovarian cancer [23]. Further analysis will be required to investigate the potential role of placental procathepsin D and cathepsin D in the pathophysiology of preeclampsia, including localization.

It has been reported that the 14kDa and 16kDa PRLs exhibit antiangiogenic and proapoptotic actions [2, 9]. An increase of 14kDa or 16kDa PRL in the placenta leads to decreased angiogenesis or increased apoptosis, causing a hypoxic atmosphere. As a result, cathepsin D is activated and production of PRL fragments is increased. Such conditions are probably associated with various complications, including non-reassuring fetal status, which is caused by the placental dysfunction that frequently coexists with PIH, intrauterine growth restriction and placental abruption.

The present study suggests that the placenta is a

source of PRL fragments. In addition to this study, other studies have reported that expression of the 14 or 16kDa PRL is increased in the urine or amniotic liquid in preeclampsia cases, and that these increases may be due to renal glomerulus and deciduas [6, 7]. In addition to PIH or preeclampsia cases, the possibility of production of PRL fragments in retinopathy, in pituitary adenoma and in the hearts of postpartum cardiomyopathy has been reported [9, 15, 24]. In several fetal and maternal tissues, including deciduas, placenta and kidney, the PRLs upregulated during pregnancy are cleaved and processed to PRL fragments, and these fragments act as antiangiogenic and proapoptotic factors, resulting in being associated with the expression of the disease state and the symptoms related to various PIH. Because this study had a small sample size and only observed the placental expression of PRL and its fragments, further studies will be needed to investigate the association of antiangiogenic fragments of PRL with the severity of the PIH and the potential roles of PRL and antiangiogenic fragments in the placenta of PIH cases.

In conclusion, PRL fragments were shown to be increased in the placenta of PIH cases, and the placenta was suggested to be the source of the increase. Although further *in vitro* and *in vivo* study is necessary, we speculate that PRL fragments are associated with the pathophysiology of the placenta dysfunction in PIH cases.

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