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The Dynamics of Cullin-1 Expression in Preeclamptic Placenta and its Association with Pregnancy Termination Time

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Abstract

BACKGROUND: Preeclampsia is a systemic syndrome occurring in 3–5% of pregnancies, caused by disorders of cellular factors resulting in the disruption of trophoblast differentiation and invasion which is important for the placental development and maintaining pregnancy. Cullin-1 is a protein that plays a role in the process of maintaining pregnancy, development, and trophoblast invasion in the placenta. Until now, there have been no studies linking the expression of cullin-1 in preeclamptic patients with the timing of pregnancy termination.

AIM: This study analyzed cullin-1 expression in preeclamptic patients and their relationship to the timing of pregnancy termination was carried out.

METHODS: Placental samples were taken from preeclampsia patients consisting of three gestational age groups, then immunohistochemical staining was performed to see the dynamics of expression and distribution in each age group of pregnancy and to find out their relationship with the timing of pregnancy termination.

RESULTS: Cullin-1 was expressed in syncytiotrophoblasts and cytotrophoblasts. The lowest cullin-1 level was obtained in the very preterm age group, and the highest was found in the moderate preterm gestational age group. There was a significant difference between cullin-1 optical density (OD) expression and termination time of pregnancy, and there was a significant difference (OD) in cullin-1 preeclamptic patients with very preterm gestational age with moderate preterm gestational age.

CONCLUSION: Cullin-1 was expressed both in syncytiotrophoblasts and cytotrophoblasts and was associated with the timing of pregnancy termination.

Introduction

Preeclampsia is a systemic syndrome occurring in 3–5% of pregnancies [1], [2]. Preeclampsia is characterized with new onset of hypertension and proteinuria by 20 weeks of pregnancy [3]. There are many cellular factors involved in regulation of placental growth which is considered as the cause of this disorder [4], [5], [6]. Recent studies were focusing on the relationship between abnormal placenta, trophoblast development, and preeclampsia. In the early stages of human placental development, there are two kinds of trophoblast, namely, villous trophoblast and extravillous trophoblast (EVT). Villous trophoblast consisted of cytotrophoblasts which fused to form syncytiotrophoblast. Syncytiotrophoblast is responsible for secreting hormones such as including human chorionic gonadotropin (hCG), a very important hormone to prevent involution of corpus luteum and maintaining secretion of progesterone from ovarian granulosa cell and human placental lactogen (hPL), a potent glycoprotein with an increasing level up to 36 weeks of pregnancy and has regulatory role on carbohydrate and lipid metabolism. There are many functions of cytotrophoblast, including nutrition and gas exchange for fetal development, regulating immune tolerance, and preserving pregnancy. EVT, comprising endovascular and interstitial EVT, is a motile and highly invasive cell from trophoblast which is undergoing epithelial–mesenchymal transition (EMT). EVT will invade endometrium and then penetrate into tunica intima of spiral artery, forming fetomaternal circulation [7], [8], [9].

Cullin-1 is a hydrophobic protein which involved in scaffolding of ubiquitin ligase (E3) enzyme. Cullin-1 alone has no catalytic activity, it has the role of scaffolding E3 ligase enzyme, moving ubiquitin from conjugate of E2 enzyme to the substrate [7]. Loss of cullin-1 in trophectoderm layer is thought to inhibit cell cycle in entering S phase due to inavailability of ubiquitinizing substance for cell cycle inhibitor p27. In relation to this phenomenon, endoreduplication of trophoblastic cells will be hindered, causing interference in placental development [10]. Another study showed that cullin-1 protein knockout, cullin-$1^{-/-}$ is causing

early death of rat embryo and disturbs cyclin-G1 and cyclin-E [11].

Severity of preeclampsia and possibility of preserving pregnancy until full term is thought to have relation with the success of trophoblastic cell. Cullin-1 is a candidate protein as the cellular factor involved in trophoblast development. However, there is no evidence whether cullin-1 is related with the period of pregnancy that can be reached by preeclampsia patient. To find the association between cullin-1 and pregnancy termination time, this study was to analyze the dynamics of cullin-1 expression in placental tissue of preeclamptic patient. Gestational age was divided into three categories, <32 weeks (very preterm), 32–37 weeks (moderate preterm), and more than 37 weeks (full term).

Methods

This study was conducted from July 2017 to December 2017 in RSUPN Dr. Cipto Mangunkusumo (RSCM) and Faculty of Medicine, University of Indonesia. The study samples were taken from vaginal delivery and cesarean section in Obstetrics and Gynaecology Department, RSCM-FK UI. Histological sample preparation, immunohistochemistry staining, sample observation, and data taking were done in anatomical pathology laboratory, Anatomical Pathology Department, Faculty of Medicine, University of Indonesia.

Study samples

Study samples were placental tissue from preeclamptic patient which were taken immediately after cesarean or vaginal birth. There were 51 samples which are divided into three categories of gestational age as follows, very preterm (n:16), moderate preterm (n:18), and full term (n:17). Sample was taken by separating one cotyledon in the periphery of placenta using sterile scissors and then washed from blood using phosphate buffer saline (PBS). Then, cotyledon was cut into some parts by 1 cm × 1 cm × 1.5 cm size using scissor and placed into a bottle which containing 10% buffer formalin.

Immunohistochemical (IHC) staining

Paraffin block was cut using microtome, then it is moved to warm water inside the water bath. After the samples were developed well, the results then placed on the object glass. IHC staining are as follows: The sample was deparaffinized with xylol I for 10 min, xylol II for 10 min, xylol for 10 min, absolute alcohol for 5 min, 95% alcohol for 5 min, 80% alcohol for 5 min, 70% alcohol for 5 min, and aquadest for 5 min. Then, sample was given blocking endogenous peroxidase (3% H_2O_2 in methanol) for 5 min followed by washing it using PBS at pH of 7.4 twice, for 5 min each. Sample then introduced with antigen retrieval solution and sodium citrate buffer (pH 6.0) and inserted into the pressure cooker for 45 min at a temperature of 95°C. Next, sample was cooled in temperature room for 30 min. Afterward, the specimen was washed twice using PBS (pH 7.4) 5 min, respectively. Non-specific activity was blocked with background sniper for 15 min. Incubation of the antibody is the next step, and it is done for one night. After incubation process, sample was washed using PBS (pH 7.4) twice for 5 min separately. Thereafter, incubation with TrekAvidin-horseradish peroxidase was done for 15 min. Next step is washing the specimen twice using PBS (pH 7.4) 5 min, respectively, and followed by amino ethylcarbazole for 5 min and washing process is repeated. Counterstaining was done using hematoxylin for 5 s. The specimen was washed using tap water, then is entered to dehydration process using increasing concentration of alcohol (70%, 80%, and 96%, absolute alcohol), 3 min, respectively, xylol I, II, and III, for 5 min each and finished with Entellan application. Preparation then observed using the microscope.

Data analysis

Cullin-1 (Cul-1) expression was evaluated descriptively for its distribution location according to the observation using light microscope and its expression power was shown in optical density (OD) unit calculated by ImageJ software. That software can evaluate pixel, presence percentage (contributions) resulting in semi-quantitative score (high positive, positive, low positive, negative). Data were analyzed with the Statistical Package for the Social Sciences (SPSS) version 20.0. Sample characteristics are displayed descriptively in the table. Normality test was done using Kolmogorov–Smirnov and followed by Levene's test for homogeneity of the data. Average score from every variable was compared using one-way ANOVA test; on the other hand, differences in Cul-1 expression in three categories of gestational age were tested using Chi-square.

Results

Placental histological image in the preeclamptic patient with gestational age of less than 32 weeks (very preterm) showed fewer villous appearances compared to moderate preterm and full-term gestational age. Cytotrophoblast cell characterized by rounded nucleus, clear margin, with eosinophilic cytoplasm in addition to small and flat syncytiotrophoblast cell. Inside the

villi, there are capillary vessels with various sizes, mostly located in central position and there are two layers of trophoblastic cells which is covering villi surface, cytotrophoblast cell as inside layer, and syncytiotrophoblast cell as outside layer, margin of syncytiotrophoblast cell and other cells are not really clear, multinucleated nucleus and grape-like cell cluster can be observed. Syncytiotrophoblast cell has direct interaction with intervillous space where mother's blood can be found, downside layer has proliferative type cytotrophoblastic cell inside the placenta (Figure 1a).

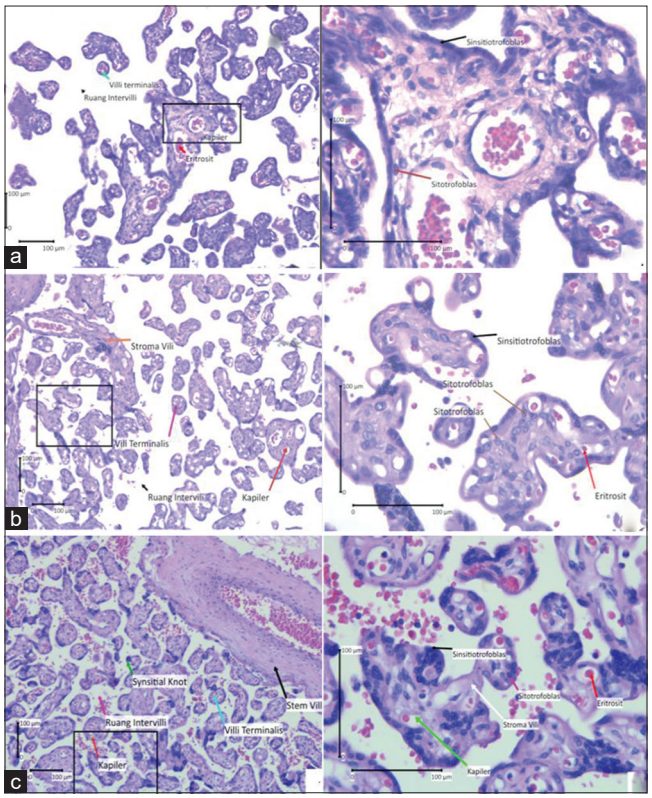

Figure 1: Histological appearance of hematoxylin and eosin staining in preeclamptic patient; (a) very preterm gestational age, (b) moderate preterm gestational age, (c) full-term gestational age

Histopathological appearance of the preeclamptic patient placenta with moderate preterm categories showed increasing villi amount compared to very preterm group, with thinner syncytiotrophoblast layer. Cytotrophoblast cell is fewer, bigger villous capillary vessel which is more periphery located, some syncytiotrophoblast cell also decreasing in amount and cannot be seen in some villi (Figure 1b). On the other hand, histological image of the preeclamptic patient with full-term gestational age showed many villi, more peripherally located fetal blood vessel. Syncytiotrophoblast cell is characterized with flat cells, multinucleated, some is showing grape-like clusterization and fewer than very preterm categories. Although cytotrophoblast is found in lesser amount, they still have function as pioneer cell for another type of later trophoblastic cell type, sometimes big trophoblastic cell with the multinucleated nucleus and syncytial knots in the periphery of villi can be found (Figure 1c).

IHC staining using cullin-1 antibody in placental tissue of the preeclamptic patient at different gestational ages classified as positive when OD score in plugin IHC Profiler ImageJ is positive (4/3/2 score) and confirmed with direct observation on trophoblast cell which is stained as brown. Positive control tissue was used for cullin-1 expression in cervical cancer. In positive control (cervical cancer), cullin-1 was stained well in nucleus or cytoplasm. It is used as the detection whether the procedure is appropriate. Trophoblast cell which is located in villi at very preterm gestational age expresses a low level of it in cytoplasm (Figure 2a). It is shown by IHC OD ImageJ average score at 1.933 (negative) (Figure 3). Very preterm gestational age group had the lowest average IHC OD score compared to other gestational age groups.

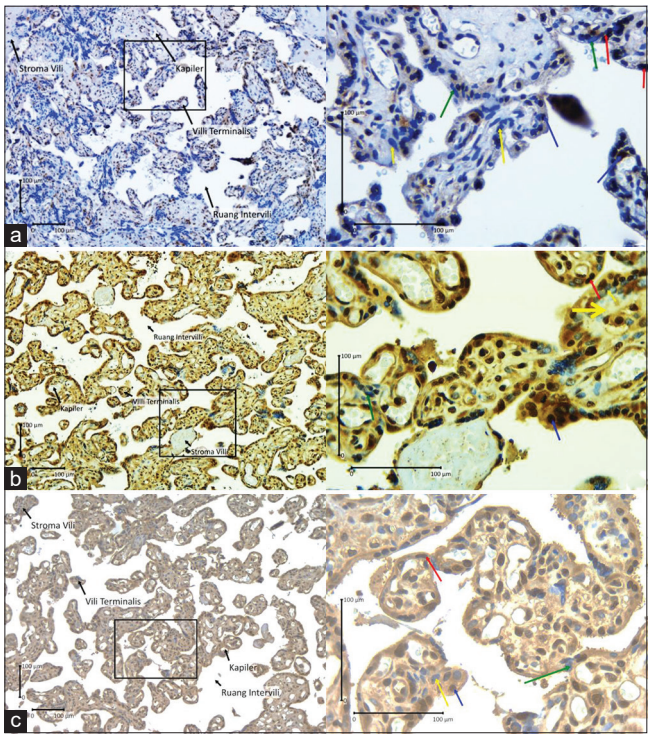

Figure 2: Cullin expression in trophoblast cell in many different gestational age group for preeclamptic patient; (a) preterm gestational age (<32 weeks), (b) gestational age 32–37 weeks, (c) gestational age more than 37 weeks.

: Cullin-1 expressing cytotrophoblast cell
: Cytotrophoblast cell which is not expressing cullin-1
: Cullin-1 expressing syncytiotrophoblast cell
: Syncytiotrophoblast cell which is not expressing cullin-1

Cullin-1 expression in the moderate preterm group of gestational age (Figure 2b) had highest average OD score in trophoblast cell villi compared to other gestational age groups. It can be seen from average IHC OD ImageJ score at 2.213 (weak positive) (Figure 3). Cullin-1 was expressed in cytoplasm of trophoblast and villous syncytiotrophoblast cell, in some parts of trophoblast villi, cullin-1 was strongly expressed, meanwhile, another area expressed weak signal of even no signal of cullin-1 (negative).

Cullin-1 in full-term group (Figure 2c) had average OD score at 2.157 (weak positive) (Figure 3).

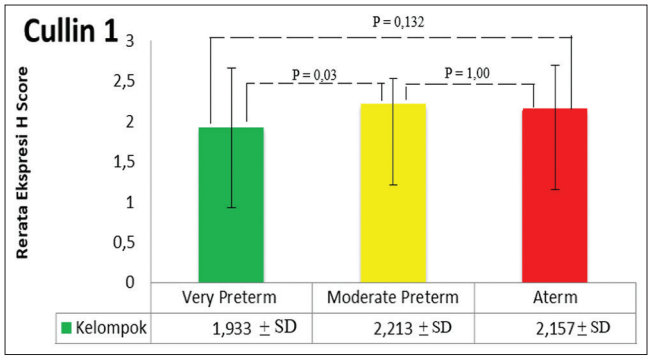

Figure 3: Average optical density value in the different gestational age group according to H score calculation

That value is higher than very preterm group, but lower than moderate preterm group.

OD data from all individuals were analyzed using one-way ANOVA test. From one-way ANOVA analysis, there are significant differences of cullin-1 expression in different gestational age groups (p = 0.03). One-way ANOVA analysis then followed by *post hoc* Bonferroni (α: 0.05). *Post hoc* test showed that very preterm and moderate preterm gestational age group had significant differences (p = 0.035); meanwhile, there are no significant differences between moderate preterm and full-term gestational age group (p = 1.000) (Figure 3).

Histological appearance of preterm placenta from the preeclamptic patient showed less villi, cytotrophoblast (big cell, round nucleus, clear margin, and eosinophilic cytoplasm) and syncytiotrophoblast (small and flat cell, capillary villi located more centrally than peripherally) were identified. Histological appearance of moderate preterm placenta from preeclamptic patient showed small increase in villi amount, less cytotrophoblast with more peripherally located, and bigger capillary in addition to less syncytiotrophoblast. Histological appearance of full-term placenta from the preeclamptic patient showed many villi, more peripherally located blood vessel and syncytial knots in villi edge.

Cullin-1 was weakly expressed in very preterm gestational age or preeclamptic patient, cullin-1 was strongly expressed in moderate preterm and full-term gestational age of preeclamptic patient.

Highest OD value was found on moderate preterm gestational age group, lowest OD score was found on very preterm gestational age group. One-way ANOVA analysis showed significant result (p = 0.03 < α 0.05), then *post hoc* Bonferroni showed significant differences between very preterm and moderate preterm group.

Discussion

Cullin-1 expression with lowest average IHC OD score was found on very preterm gestational age group. Low level of cullin-1 could lead into the increased probability of developing preeclampsia [10]. Cullin-1 is a hydrophobic protein acting as the scaffold for ubiquitin ligase (E3) enzyme. Every single eukaryote possess this kind of protein, combined with RING protein to forms cullin-RING ubiquitin ligase (CRLs cullin-based ubiquitin ligases) [12], [13]. Cullin-1 single handedly has no catalytic enzyme activity; on the other hand, it acts as scaffolding for enzyme E3 ligase, moving ubiquitin from conjugate of E2 enzyme to its substrate [14]. Through ubiquitination process, CRLs/stem cell factor (SCF) acts in many cellular processes, including cell cycle, transcription, signal transduction, and embryogenesis [15]. Earlier studies report that knockout of cullin protein, cullin-$1^{-/-}$ is causing premature death of rat embryo and disturbance of cyclin-G1 and cyclin-E [16].

The past studies showed that F-Box SKP2 protein which was arranged in SCF-SKP1 complex was targeting the destruction of cell cycle inhibitor, p27 that could bring the cell to enter S phase [17]. Loss of cullin-1 in trophectoderm layer was thought to inhibit the cell cycle in entering S phase. This event could lead into endoreduplication of trophectoderm cell. Some substrates which had contact with SCF are directly linked to modulate cyclin-dependent kinases (CDK) activity, three F-box adaptor proteins were associated with SCF-related cell cycle. Skp2 level will increase along transition of G1-S phase as the result of anaphase promoting complex/cyclosome (APC/C Cdh1) inactivity. SCF-Skp2 actively had some function, such as mediating destruction of CKIs p27Kip1, p21Cip1, and p57kip2 and pocket protein p130/RB2, increasing cyclin phase S/CDK activity, and permitting cells to go through S and G2 phase of cell cycle [18].

Along with cell cycle process, endoreduplication of trophoblast cell is happening, amount of trophoblast cell will increase and work as its function. Trophoblast cell is progenitor cell that could differentiate into two main pathways, villous trophoblast and extravillous invasive trophoblast [8], [9]. Extravillous cytotrophoblast, including endovascular and interstitial EVT, is a motile and very invasive cell from CTBs which are undergoing EMT. EVT will invade endometrium and penetrate intima of spiral artery to make fetomaternal circulation, an important factor of implantation [19], [20]. Trophoblast invasions are an important process in placental development. EVT, including interstitial and endovascular EVT, is very motile and invasive. They are entering endometrium to place the placenta into it [12]. Villous trophoblast also has important role in pregnancy. It will differentiate by fusion to make multinucleated syncytiotrophoblast which is covering chorionic villous, protecting all surface of developing placenta, and forming separating wall between fetal and mother's blood. This wall has important function, especially in the 1st week of pregnancy, besides for nutrition exchange it also protecting fetus from immunological attack from mother's tissue [21], [22].

Syncytiotrophoblast is responsible for the production of some placental hormones, especially hCG, a really important hormone to prevent involution of corpus luteum and preserving progesterone secretion by ovary granulosa cell. Syncytiotrophoblasts villi are also producing hPL, potent glycoprotein which has increasing level up to 36 weeks of pregnancy, HPL is responsible for the regulation of carbohydrate and lipid metabolism. Syncytiotrophoblast is also producing prolactin, relaxin, adrenocorticotropin, and protein-like ACTH [23]. Syncytiotrophoblasts have many functions, such as nutrition and gas exchange for fetal development, regulating immunologic tolerance and preserving pregnancy [8], [9].

Terminating pregnancy to evacuate the placenta is preeclampsia therapy nowadays, because the placenta is considered as the source of preeclampsia pathogenesis [4], [5], [6]. This study showed that cullin-1 expression (stated with OD) is lowest in the youngest gestational age group (<32 weeks, very preterm) and has significant differences with other group (32–37 weeks, moderate preterm). Preeclampsia in early stages of pregnancy showed disturbance of pregnancy, one of them are caused by low level of cullin-1 in that gestational age, it is evidenced in this study as expressed with lowest OD in very preterm gestational age group. Statistical analysis using ANOVA shows significant differences; meanwhile, *post hoc* Bonferroni test showed significant differences between very preterm gestational age and more advanced gestational age (moderate preterm).

Conclusion

Cullin-1 was expressed in cytotrophoblast and syncytiotrophoblast cell, where lowest OD of cullin-1 was found on very preterm gestational age and highest cullin-1 OD was found on moderate preterm gestational age. One-way ANOVA analysis showed that there were significant differences between cullin-1 OD of different groups of gestational age, which showed association between cullin-1 OD and termination of pregnancy in many different groups of preeclamptic patients.

Authors' Contributions

Authors equally contributed to design, data compiling and analysis, and the composing of the manuscript.

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