福岡大医紀（*Med. Bull. Fukuoka Univ.*）：35(3), 137–143, 2008

Analysis of the Proliferative Potential of Trophoblasts throughout Pregnancy

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Abstract：Trophoblasts play a pivotal role in the development and maintenance of the feto–placental environment during pregnancy. As some trophoblasts proliferate and the others differentiate in the three trimesters of gestation, trophoblasts contribute to a variety of biological processes including nutrition, circulation, and protection. The aim of this study was to investigate the proliferative potential of trophoblasts in the three trimesters of gestation, using flow cytometry and immunohistochemical staining for proliferating cell nuclear antigen (PCNA) and silver staining of nuclear organizer regions(AgNORs). Flow cytometric analysis revealed a significant decrease in the proliferative index as the gestational period progressed, suggesting that the number of proliferating cytotrophoblasts may gradually decrease in pregnancy. During pregnancy, villous cytotrophoblasts as well as anchoring villous cytotrophoblasts were positively stained with antibody against PCNA, whereas villous syncytiotrophoblasts and intermediate trophoblasts displayed negative staining. No significant difference in the number of positively–stained trophoblasts was found between the first and third trimester. In silver staining for NORs, the number of dots per cytotrophoblast nucleus was significantly increased, compared to that of syncytiotrophoblasts, both in the first and the third trimesters. In addition, the area per dot of AgNORs in the cytotrophoblasts was significantly reduced, compared to that in syncytiotrophoblasts, both in the first and third trimesters. However, temporally there were no significant differences throughout pregnancy in the number of dots per nucleus or area per dot of AgNORs in either cytotrophoblasts or syncytiotrophoblasts. Taken together, these results suggest that trophoblasts may restore proliferative properties throughout pregnancy.

Key words：Pregnancy, Trophoblast, Proliferation, Intrauterine growth restriction (IUGR).

Introduction

Trophoblasts are of fetal origin and represent a very unique tissue type, exerting a crucial role in the maintenance of pregnancy, including implantation and placentation. Villous trophoblastic tissues consist of two layers；cytotrophoblasts and syncytiotrophoblasts. Human chorionic gonadotropin and human placental lactogen, the two major hormones for the maintenance of pregnancy, are exclusively localized in syncytiotrophoblasts from 6 weeks gestation onwards.[1] However, immunohistochemical studies using various proliferative markers revealed that the proliferative potential of syncytiotrophoblasts is low throughout the whole cell cycle, in early as well as late pregnancy, compared to cytotrophoblasts.[2)3)] It is thought that the villous cytotrophoblasts, which are the precursor cells of syncytiotrophoblasts, play a key role in the overall trophoblastic function. In pathological settings, however, this unique characteristic of trophoblasts is impaired. In preeclampsia, for instance, the cellular activity

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of cytotrophoblasts is paradoxically increased compared to that in normal pregnancy, possibly due to a compensation for impaired placental function.[4)5)]

The aim of this study was to analyze the proliferative potential of trophoblasts in normal pregnancy and intrauterine growth restriction (IUGR) by flow cytometry and immunohistochemical methods using proliferative cell nuclear antigen(PCNA)/cyclin and silver-staining of nuclear organizer regions (AgNORs) as proliferation markers.

Materials and Methods

Tissue sampling

Chorionic villi were sampled from 10 cases of normal singleton pregnancy who underwent hysterectomy or had an induced abortion at 5–14 weeks' gestation (*early group*), 10 cases of spontaneous abortion or premature delivery at 17–36 weeks' gestation(*mid group*), and 10 cases who underwent elective cesarean section at 37–41 weeks' gestation(*term group*). None of these cases were smokers, and were not on any kind of medication except for iron and vitamin supplements. All fetuses in the mid and term groups were without any superficial anomalies and were appropriate for the particular stage. In addition, tissue was sampled from 10 cases of IUGR at 33–38 weeks' gestation without overt fetal malformations. Samples were obtained at the Department of Obstetrics and Gynecology, Fukuoka University Hospital. All specimens were fixed in 10% formaldehyde for 24 hours and embedded in paraffin until further analysis.

Flow cytometry

Measurements of the cellular DNA content of paraffin-embedded tissues were performed as previously described.[6)] Paraffin-embedded tissues were cut at 50 μm thickness, and the sections were placed in 10 ml glass centrifuge tubes and dewaxed using two changes of xylene, and 3 ml of 100, 95, 70 and 50% ethanol for 10 min each at room temperature. Dewaxed tissues were washed twice in distilled water and suspended in 1 ml of 0.5% pepsin and saline, and the pH adjusted to 1.5 with 2N hydrochloride. Next, the tubes containing cell suspensions were placed in a water bath at 37℃ for 30 min with intermittent vortex mixing. Cell counting was performed using a hemocytometer. When the cell number was greater than 105, the suspension was centrifuged and the pellet was resuspended in staining solution. The data was obtained from flow cytometry reports generated from the Becton Dickinson FACScan analysis. The proliferation index was calculated using the following formula:

$$\text{Proliferation index}(\%) = (S+G_2M)/(G_0/G_1+S+G_2M)\times 100$$

The proliferation indices of the early, mid and term groups were compared. In addition, a comparison was made between the proliferation indices of IUGR and the control group. The control group consisted of 10 gestational age-matched cases randomly chosen from mid and term groups.

Immunohistochemistry of anti-PCNA/cyclin monoclonal antibody

Anti-PCNA/cyclin monoclonal antibody (American Biotech, USA) was used to detect the distribution of proliferating cells in trophoblasts. PCNA is an auxiliary 36 kDa polypeptide of DNA polymerase delta,[7)] and whose expression is associated with cell proliferation. Immunohistochemically, the PCNA antibody reacts to nuclei of late G_1 and S phase cells[8)] and its binding activity does not deteriorate in the paraffin-embedded tissues.[9)]

Paraffin-embedded chorionic villi/placenta from each group was cut at 4 μm thickness. The PCNA antibody was employed using the alkaline phosphatase-conjugated avidin-biotin complex method.[10)] Briefly, tissues were deparaffinized through graded alcohol washes, and washed in buffer A composed of phosphate buffered saline (PBS) with 0.1% bovine serum albumin (BSA). The diluted anti-PCNA/cyclin antibody was overlaid on the tissue sections for 1 hour at room temperature. After washing in buffer A, biotinylated anti-mouse IgG (Vector Laboratories, USA) was applied at a dilution of 1:200 and incubated for 30 min at room temperature. After washing again in buffer A, alkaline phosphatase-conjugated avidin (Dako, Japan) was applied to the sections at a 1:100 dilution in PBS with 1% BSA for 30 min at room temperature. The immunoreactivity of PCNA/cyclin in the chori-

onic villi/placenta was compared between the early and term groups.

Histochemistry of AgNORs

Nuclear Organizer Regions (NORs) are loops of DNA which appear in nucleoli, and the number and size of NORs reflects the cellular proliferative potential.[11] These two parameters are inversely correlated, i.e., cells having low proliferative potential exhibit fewer, but larger, stained NORs per nucleus, whereas cells having high proliferative potential show more but smaller NORs per nucleus.

Paraffin-embedded sections were cut at 4 μm thickness and dewaxed in xylene, and hydrated through ethanol to deionized water. AgNOR staining solution was prepared as follows; 1) gelatin was dissolved in 1% formic acid to a concentration of 2%, 2) One volume of the aforementioned solution was mixed with two volumes of 50% aqueous silver nitrate solution to give the final working solution. This solution was poured over the tissue sections and left for 45 min at room temperature in a light-shielded dark room. The sections were dehydrated to xylene and mounted in the synthetic medium.

As previously reported,[12] quantitative analysis

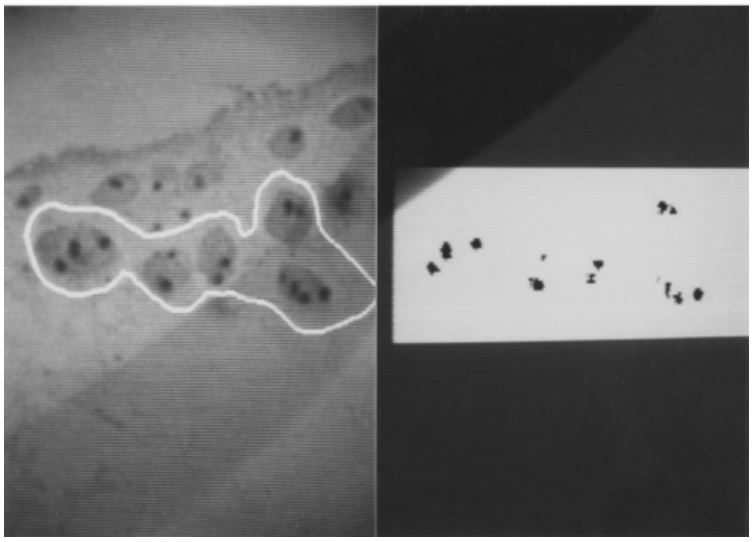

Fig. 1. Silver-stained nucleolar organizer regions (AgNORs) in the chorionic villi. The positive-staining nuclei of cytotrophoblasts were retrieved (left) and the corresponding AgNOR dots were enhanced after the background was subtracted (right).

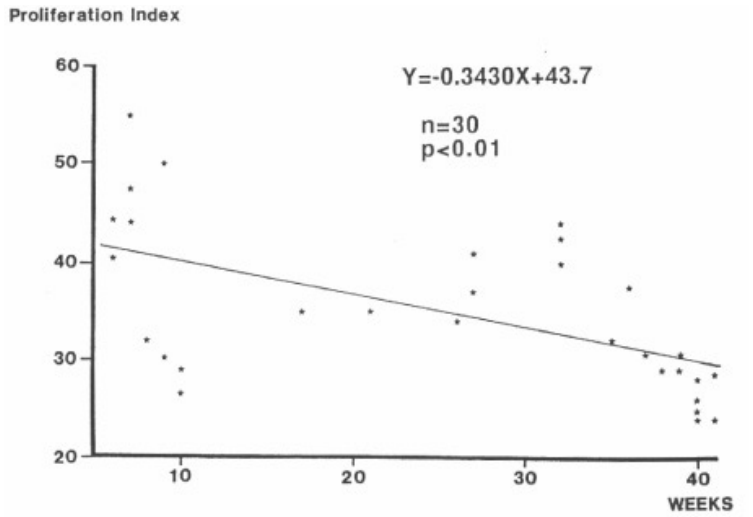

Fig. 2. Age-related changes in the proliferation index.

of AgNOR staining was performed using an image analysis system(PIAS LA555, Japan). In this system, the original image was input by video camera and the dots representing AgNORs were specifically enhanced on digital image color display (Fig. 1). After segmentation of the corresponding AgNORs was manually performed, at least 100 cytotrophoblasts and 100 syncytiotrophoblasts were examined from randomly chosen fields, avoiding non-trophoblastic areas. The number of AgNORs and the area/dots of AgNORs were compared between syncytiotrophoblasts and cytotrophoblasts in the early and term groups.

Statistical analysis

Statistical analysis was performed with the Mann-Whitney *U* test using statistical software, and the statistical significance was set at $p<0.05$.

Results

1. Proliferation index

The proliferation index of the chorionic villi decreased in a linear fashion as the gestational age advanced($Y=-0.343X+43.7$, $p<0.01$)(Fig. 2). When comparing the proliferation indices between IUGR and controls, there was no significant difference between IUGR (mean : 34.7, range : 21.0-49.0) and control (mean : 28.9, range : 24.0-42.6).

2. Immunohistochemistry with the anti-PCNA/cyclin monoclonal antibody

In the early chorionic villi, the nuclei of villous cytotrophoblasts and stromal cells were positive for PCNA, however, the nuclei of syncytiotrophoblasts were negative (Fig 3, A). Anchoring villi

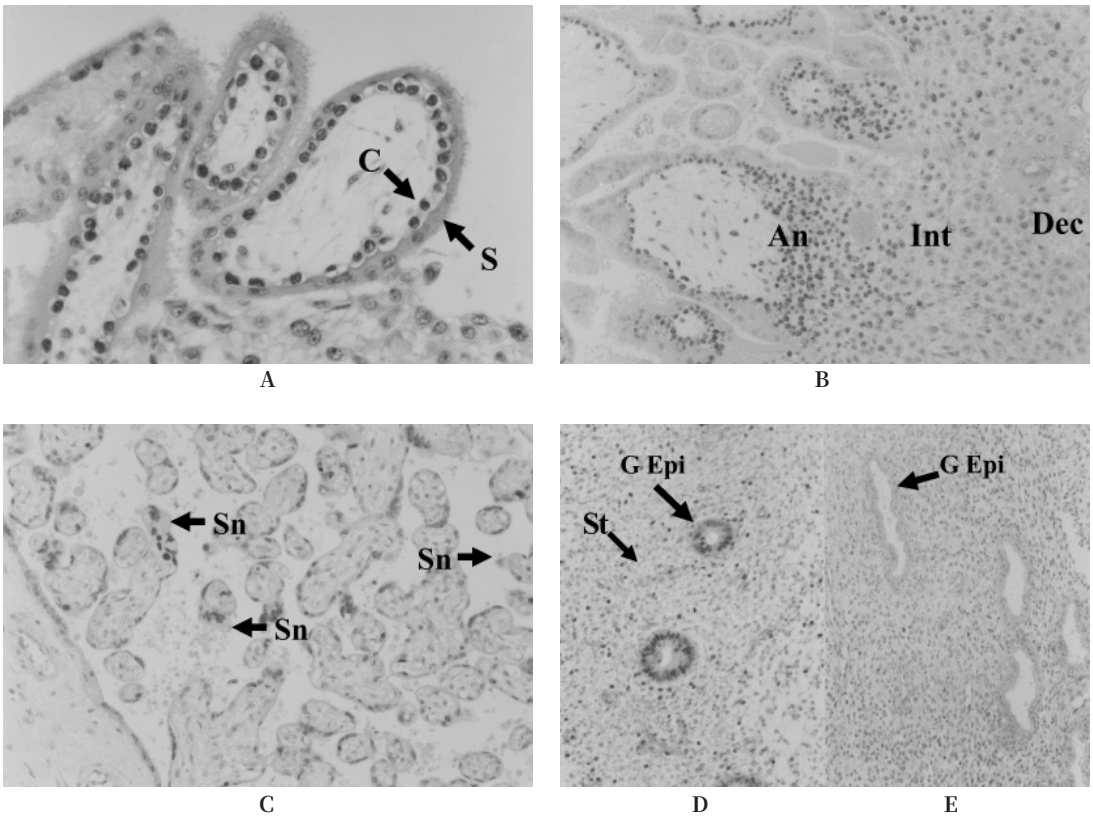

Fig. 3. Immunohistochemical localization of anti-PCNA/cyclin in the human chorionic villi at 7 weeks (A), 11 weeks (B) and 39 weeks (C) of gestation and in the human endometrium in proliferative (D) and secretory phases (E). In the proliferative phase of human endometrium, most of the glandular epithelial cells and some stromal cells were strongly positive for PCNA (positive control). In the secretory phase, endometrial glandular epithelium and stromal cells were negative (negative control).
An : anchoring villi, C : cytotrophoblast, G Epi : granular epithelial cells, Int : intermediate trophoblast, Sn : syncytial knot, St : stromal cell, T : syncytiotrophoblast.

Table 1. Number of dots and area/dots of AgNORs in cytotrophoblasts and syncytiotrophoblasts in the early and term groups.

		Cytotrophoblasts		Syncytiotrophoblasts		*P value*
No of AgNORs	*Early*	7.0(3.1–11.5)	┐ NS	5.0(2.5–9.3)	┐ NS	$p<0.01$
	Term	6.1(3.4–10.1)	┘	4.7(2.3–8.9)	┘	NS
Area/Dots of AgNORs	*Early*	0.15(0.09–0.23)	┐ NS	0.22(0.15–0.28)	┐ NS	$p<0.05$
	Term	0.17(0.09–0.21)	┘	0.20(0.15–0.27)	┘	NS

Numbers shown indicate the mean and range.

were reactive with PCNA antibody, but extravillous trophoblasts, especially intermediate trophoblasts, were not (Fig 3, B). In the term chorionic villi, villous syncytiotrophoblasts were not positive for PCNA, but underlying cytotrophoblasts were (Fig 3, C). The staining pattern was similar to that of early chorionic villi. Some prominent and typical syncytial knots were observed, however, these syncytial knots were not positive for PCNA.

3. Analysis of immunochemistry of AgNORs

In the analysis of AgNORs, the positive dots in the trophoblasts were identified in the nuclei. Syncytiotrophoblasts exhibited fewer but larger dots representing AgNORs, per nucleus. On the contrary, cytotrophoblasts demonstrated more and smaller AgNOR dots.

In the early chorionic villi, the number of dots per nucleus in cytotrophoblasts was greater than that of syncytiotrophoblasts, and the area per dot of AgNORs in cytotrophoblasts was smaller than that of syncytiotrophoblasts ($p<0.05$) (Table 1). In the term chorionic villi, the number of dots per nucleus in cytotrophoblasts was also greater than that of syncytiotrophoblasts, and the area per AgNOR dot in cytotrophoblasts was smaller than that of syncytiotrophoblasts. However, these differences were not significant.

There were no significant differences in the number of AgNOR dots, nor the area per dot, in cytotrophoblasts or syncytiotrophoblasts between the early and term chorionic villi (Table 1).

Discussion

The proliferative potential of normal and abnormal trophoblasts has been extensively studied. However, the process of proliferation and differentiation of trophoblasts still remains to be elucidated. The placental weight increases in an almost linear fashion as gestation progresses, while the rate of placental weight gain is reduced in the 3rd trimester. There have been few studies on the proliferative potential of placenta throughout pregnancy.[13]

Firstly, we analyzed the overall proliferative potential of chorionic villi. In this study, cell cycle analysis by flow cytometry revealed that the proliferation index of placenta significantly decreased as gestation progressed, indicating that the proliferative potential of placenta decreased accordingly. Bulmer et al. reported the proliferative potential of cytotrophoblasts and syncytiotrophoblasts in the early and term placenta.[14] Using Ki–67, a monoclonal antibody for proliferating cells, they demonstrated high proliferative potential of cytotrophoblasts and low proliferative potential of syncytiotrophoblasts in early as well as late gestation.

In the present study, we used anti–PCNA antibody. This antibody works on formaldehyde fixed and paraffin embedded tissue, which is advantage for the retrospective analysis. Because of the paraffin embedded sections, tissue sampling and preservation are very easier than criostat sections. In addition, paraffin embedded sections enable to make easy to observe the morphological findings. Using an anti–PCNA monoclonal antibody, we already confirmed immnunohistochemically of high proliferative potential of cytotrophoblasts and lack of proliferative potential of syncytiotrophoblasts.[15]

In this study, the results of AgNORs image analysis revealed that the proliferative potential of cytotrophoblasts was higher than that of syncytiotrophoblasts throughout pregnancy. Villous syncytiotrophoblast was not consistently reactive with PCNA but the underlying cytotrophoblast and cytotrophoblast of anchoring villi were reac-

tive with PCNA throughout pregnancy. Extravillous trophoblasts, especially intermediate trophoblasts, were not positive for PCNA. Based on the above-mentioned results, it is suggested that anchoring villi are the source of proliferation of trophoblasts. In addition, there may be two directions of cell proliferation on chorionic villi ; one to the surface epithelium of chorionic villi, and the other to the deeper portion of decidua.

Syncytial knots are thought to be histological changes as a result of prolonged local hypoxic stress in the chorionic villi. There are two hypotheses concerning the pathogenesis of syncytial knots. One is the degenerative changes in syncytiotrophoblasts following hypoxia[16)17)] and the other is compensatory proliferative changes in syncytiotrophoblasts.[18)] In this study, the nuclei of syncytial knots were unreactive with PCNA antibody. Further, this tendency is the same as in three cases with placental hypoxic change. Our results support the idea that syncytial knots are degenerative changes in syncytiotrophoblasts.

Immunohistochemically, the numbers of PCNA-positive cells decreased as gestation progressed, and this tendency seemed to be in accordance with the population of cytotrophoblasts during placental development.[19)20)] The image analysis of AgNORs indicated that the proliferative potential of cytotrophoblasts was unchanged throughout pregnancy. Cytotrophoblasts have been established as being the proliferating cells within normal chorionic villous tissue, and PCNA antibody reacted with villous cytotrophoblasts both in early pregnancy and at term. The presence of proliferating cells in late pregnancy is not surprising, since the term placenta is capable of in vitro proliferation. The proliferative potential in the placenta throughout pregnancy might depend on the number of cytotrophoblasts. Previously, an increased number of cytotrophoblasts in preeclampsia was reported.[21)] This previous report revealed that the villous cytotrophoblastic cell count is one of the most reliable parameters for the assessment of placental ischemia/infarction. In our study, the number of cytotrophoblasts seemed to be correlated with the proliferation index of placenta. We analyzed the proliferation index of chorionic villi from IUGR, however, contrary to our expectations, there was no significant difference from the controls.

Cell proliferation and differentiation are different phenomena, however, they are tightly associated with each other. Generally, previous investigators used Ki-67 or BrdU to test for cell proliferation and epidermal growth factor (EGF) or EGF receptor (EGFR) as a marker of cell differentiation. Maruo et al. reported that immunohistochemical analysis revealed that EGF localized on syncytiotrophoblasts is related to the differentiation of syncytiotrophoblasts,[1)] while other investigators showed that EGF deficiency during pregnancy caused abortion in mice.[22)] Our results indicated no proliferative potential in syncytiotrophoblasts during pregnancy, which demonstrates the functional difference between syncytiotrophoblasts and cytotrophoblasts.

In the future, comparison of the proliferative potential of trophoblasts throughout pregnancy might contribute to elucidation of the pathogenesis of perinatal disorders, including IUGR, preeclampsia and spontaneous abortion.

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(Received on April 30, 2008,
Accepted on June 12, 2008)