Changes in Placental Cell Proliferation during Pregnancy in the Rabbit

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Abstract. Changes in proliferation of rabbit placental cells during pregnancy were evaluated by bromodeoxyuridine (BrdU) incorporation into the DNA. Cells were isolated and purified from rabbit placental tissues at different stages of pregnancy (day 11, 17, 23 or 29). BrdU incorporation as detected by an ELISA, indicated that cells isolated on day 11 were mitotically active in growth factor free medium. However, BrdU incorporation decreased as pregnancy progressed; this paralleled the change in placental weight gain. These results suggest that rabbit placental growth may be regulated not only by maternal factors but also by local factors.

Key words: Rabbit, Placenta, Cell proliferation, Bromodeoxyuridine.

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The mammalian placenta grows rapidly during pregnancy. The placenta provides nutrition for the fetus, exchanges blood gases, allows disposal of fetal excretory material, and maintains pregnancy by a variety of hormonal mechanisms. It has been shown that the placenta is also involved in immunoprotection of the embryo [1]. The placenta is the most invasive and proliferative tissue when compared to other general tissues. Therefore, several studies have examined the roles of growth factors in placentation in humans [2] and mice [3].

The rabbit has a discoid placenta similar to humans and mice. However, its hemo-dichorial and labyrinthine structures differ from humans (hemo-monochorial and villous) and mice (hemo-trichorial and labyrinthine) [4]. Rabbits also have centric implantation that differs from humans which have interstitial implantation and from mice

which have eccentric implantation. Thus, the morphology of rabbit placentae appears to be specific. Accordingly, a number of morphological studies have reported on rabbit placentation [5, 6]. However, no report appears to have evaluated biochemical factors involved in rabbit placental cell proliferation.

In this study we observed chronological changes in placental cell proliferation during pregnancy by bromodeoxyuridine (BrdU) incorporation into cells isolated from placental tissues on day 11 (early stage of placentation), day 17 (late stage of placentation), day 23 (fetal stage), and day 29 (the term) of pregnancy in rabbits.

Materials and methods

Materials

Ca²⁺-Mg²⁺ free Hanks' balanced salt solution (HBSS), Dulbecco's modified Eagle's medium

(DMEM) and calf serum were obtained from GIBCO (Grand Island, USA). Trypsin type III, collagenase type IV, deoxyribonuclease I (DNase I), bovine serum albumin (BSA), and 5-bromo-2'-deoxyuridine (BrdU) were from Sigma Chemical (St. Louis, USA). Percoll was from Pharmacia (Uppsala, Sweden). Tissue-Tek OCT compound was from Miles (Elkhart, USA). Monoclonal anti-cytokeratin cocktail (MAK-6) was from Triton diagnostics (Alameda, USA). Biotinylated horse anti-mouse IgG, avidin DH, and biotinylated horseradish peroxidase H reagents were from Vector Laboratories (Burlingame, USA). Flat-bottomed 96-well tissue culture plates were from Flow Laboratories (McLean, USA). Benzylpenicillin potassium and streptomycin sulfate were from Meiji-Seika (Tokyo). Pepsin was from Biozyme Laboratories (Blaenavon, UK). Anti-BrdU monoclonal antibody was from Immunotech (Marseille, France). Diaminobenzidine tetrahydrochloride (DAB) and other reagents were from Wako Pure Chemical (Osaka).

Animals

Japanese White female rabbits aged 4 months (3.3–4.0 kg body weight) were purchased from Kitayama Labes (Nagano) and mated with males of the same strain. The day of copulation was confirmed by the presence of spermatozoa in a vaginal smear and defined as day 0 of pregnancy. The rabbits were housed individually under controlled light (0500–1900 h), temperature ($23 \pm 2^\circ\text{C}$) and relative humidity ($55 \pm 10\%$) and given free access to solid chow (RC4; Oriental Yeast, Tokyo) and water.

Placental cell isolation

Rabbits were euthanized by CO_2 inhalation on day 11, 17, 23, or 29 of pregnancy. Live fetuses and labyrinthine zones of placentae were obtained after dissection and weighed immediately. Placental cell isolation was based on the procedure of Kliman *et al.* [7]. HBSS-washed placental tissue (0.3–2.2 g) was minced and digested in a twenty-fold volume (vol/wt) of HBSS containing 0.25% trypsin and 0.25% collagenase for 20 min at 37°C with shaking. The cell suspension was filtered through 70- μm mesh and centrifuged at $250 \times g$ for 5 min. The pellet was suspended in 5 ml of HBSS containing 20% heat-inactivated calf serum and 2 mM EDTA to inhibit enzyme activity and

centrifuged at $250 \times g$ for 5 min. The resulting pellet was resuspended in 4 ml of DMEM containing 50 Kunitz units/ml DNase I, layered on a discontinuous Percoll gradient [70% (2 ml), 50% (2 ml), 30% (1 ml) and 20% (2 ml) Percoll diluted with HBSS], and centrifuged at $1,200 \times g$ for 20 min. The cells concentrated between densities 1,035 and 1,062 were removed and washed with HBSS and centrifuged at $250 \times g$ for 5 min. The final pellet was resuspended in 1–3 ml of DMEM containing 50 Kunitz units/ml DNase I. Live cells were counted by trypan blue exclusion with an improved-Neubauer hemacytometer (Becton Dickinson, Buffalo, USA).

Immunohistochemistry

Isolated placental cells were fixed with 2% formaldehyde for 30 min at 4°C and air-dried on glass slides. The uterus obtained from a day 14 pregnant rabbit was fixed with periodate-lysine-paraformaldehyde fixative [8] for 20 h at 4°C and washed with PBS containing sucrose (10–30%). Implantation sites were removed from the uterus, embedded in OCT compound, frozen and stored at -80°C . Cryostat sections (5 μm) were mounted on glass slides and air-dried.

The cell preparation was immersed in 0.5% Triton X-100 in PBS for 30 min to permeabilize cell membranes. Cell and uterine tissue preparations were incubated with 1.5% normal horse serum in PBS for 10 min, MAK-6 for 10 min, biotinylated horse anti-mouse IgG diluted 1/200 in PBS for 10 min, 3% H_2O_2 in methanol for 3 min, avidin biotin peroxidase complex reagent for 10 min, and 0.05% DAB and 0.01% H_2O_2 in 0.1 M Tris-HCl buffer (pH 7.2) for 5 min at 25°C . The preparations were counterstained with methylgreen. Control preparations were incubated with 1.5% normal mouse serum in PBS in place of MAK-6.

BrdU incorporation

The placental cells were placed in 100 μl /well of DMEM that contained 50 Kunitz units/ml DNase I, 100 IU/ml penicillin, and 100 μg /ml streptomycin in the 96-well plates and were incubated in humidified 5% CO_2 -95% air at 37°C . Twenty-four hours later, BrdU was added to the medium, and the incubation was continued. The incorporated BrdU was detected by ELISA [9] as follows: At the end of incubation, the cells were washed twice with PBS by centrifugation of the plates at $750 \times g$ for 2

min. After air-drying the plates, the cells were fixed with 200 μ l/well of ethanol and air-dried again. The fixed cells were then incubated with 5 mg/ml pepsin in 0.1 N HCl for 30 min at 37 C and, subsequently, with 4 N HCl for 20 min at 25 C to denature the DNA. Following 2 washes with 250 μ l/well of 0.1 M $\text{Na}_2\text{B}_4\text{O}_7$ and three washes with 250 μ l/well of PBS, the plates were incubated with 1% BSA in PBS overnight at 4 C for blocking. The blocking solution was discarded, and the plates were incubated with 100 ng/ml anti-BrdU monoclonal antibody in PBS, biotinylated horse anti-mouse IgG diluted 1/200 in PBS, and avidin biotin peroxidase complex reagent for 10 min each on ice. The plates were washed 3 times with 0.1% Tween 20 in PBS, and 100 μ l/well of substrate solution consisting of 0.4 mg/ml *o*-phenylenediamine and 0.009% H_2O_2 dissolved in 0.1 M citric acid- Na_2HPO_4 buffer (pH 5.0) was added. After incubation for 20 min at 25 C, the reaction was terminated by adding 25 μ l/well 8 N H_2SO_4 . Absorbance was measured by reading the plates at 492 nm in a Titertek Multiskan MCC (Flow Laboratories, McLean, USA). All measurements were made in triplicate.

Optimal conditions for detecting incorporated BrdU were examined by varying exposure times (1, 5, 24 h), BrdU concentrations (10^{-5} – 10^{-3} M), and cell counts (5000–80000/well) using placental

cells prepared from a specimen on day 11 of pregnancy. Based on these results, 20000 cells/well incubated with 10^{-4} M BrdU for 24 hours were found to be optimal and, hence, were used in the present experiment.

Statistical analysis

The absorbance values were analyzed among days of pregnancy by one-way analysis of variance followed by the least significant difference method. $P < 0.05$ was considered to be significant.

Results

Changes in fetal and placental weights are given in Fig. 1. The gain in placental weight reached plateau in the late stage of pregnancy in contrast with fetal growth that continued to the end of pregnancy. Viability of the collected cells, based on trypan blue exclusion, was greater than 90%. Yield of live cells per 1 g wet weight of placental tissue was between 7.72 (day 11) and 4.84 (day 29) $\times 10^6$. The yield tended to decrease as pregnancy progressed, but the difference was not significant among these days of pregnancy. The majority of collected cells were mononuclear with a 10–20 μ m diameter (Fig. 2). There were polynuclear and giant cells with a diameter of more than 30 μ m, but they were less than 5% of total cell count. These morphological characteristics did not change from day 11 to 29 of pregnancy.

Immunohistochemical staining of uterine tissue

Fig. 1. Changes in fetal and placental weights during pregnancy in rabbits. All data represent the mean $\pm$ SD of four pregnant rabbits.

Fig. 2. Cells dispersed from rabbit placenta on day 11 of pregnancy, concentrated between densities 1.035 and 1.062 of Percoll gradient, fixed with 2% formaldehyde, and stained with hematoxylin-eosin. Magnification, $\times 426$.

with cytokeratin antibody revealed positive reactions with the fetal placenta, such as the placental labyrinth and junctional zone, but a negative reaction occurred in the decidua (Fig. 3a). Cells isolated from day 11–29 placentae also showed positive staining in all cells observed including the giant cells (Fig. 3c).

A linear correlation between BrdU exposure time and absorbance was noted up to 24 h in day 11 placental cells (Fig. 4a). The absorbance linked to BrdU concentration increased linearly from 10^{-5} to 4×10^{-5} M then reached its plateau at 10^{-4} M (Fig. 4b). Absorbance was also dependent on cell count; it increased linearly from 5000 to 20000 cells/well and then reached its plateau at 40000 cells/well (Fig. 4c). According to these results, 20000 placental cells per well were incubated with 10^{-4} M BrdU for 24 hours to compare BrdU incorporation among days 11, 17, 23, and 29 of pregnancy. The cells on day 11 showed the greatest incorporation of BrdU with 1.12 ± 0.08 (mean $\pm$ SD, $n=4$) absorbance at 492 nm. BrdU incorporation decreased to 0.40 ± 0.04 on day 17 and then to 0.25 ± 0.03 on day 23. Finally, the cells on day 29 did not show measurable incorporation of BrdU. Thus, BrdU incorporation decreased with the progress of pregnancy (Fig. 5), and the differences were statistically significant among all days observed.

Discussion

The placenta in rabbits appears on the decidua on day 9 of pregnancy [5]; it then develops and completes its hemo-dichorial labyrinth by day 18 [6]. Changes in placental weight show that placental growth in rabbits is rapid up to day 17 of pregnancy but reaches plateau after day 23 in contrast to fetal growth. This finding is consistent with a morphological observation of rabbit placentation by Kotera [6].

Iguchi *et al.* [10] reported that trophoblastic cells from murine placentae on day 6 to 18 of pregnancy increased in number when cultured in medium containing 10% fetal bovine serum for 18 days but did not proliferate in serum free medium. Cell proliferation in culture requires specific conditions, and, hence, medium composition and cell attachment vary for each type of cells. Accordingly, we assessed the proliferation potency of rabbit placental cells using BrdU incorporation into the DNA as an index of DNA synthesis. Despite the absence of growth factor in the medium, the isolated rabbit placental cells on day 11 of pregnancy actively incorporated BrdU into the DNA. The incorporated BrdU decreased with the progress of pregnancy, which in turn, appears to be related to the change in placental weight gain.

Fig. 3. Placental tissue and cells stained immunohistochemically with cytokeratin antibody. a) Implantation site of day 14 pregnant rabbit. Placental labyrinth (P) shows positive, but decidua (D) shows negative. Magnification, $\times 43$. b) Negative control treated with 1.5% normal mouse serum. Magnification, $\times 43$. c) Cells from a placenta on day 11 of pregnancy show positive. Magnification, $\times 426$.

Fig. 4. Effect of a) BrdU exposure time, b) BrdU concentration and c) cell count on BrdU incorporation into isolated rabbit placental cells on day 11 of pregnancy. The cells were preincubated in DMEM for 24 h. Basal conditions were 10^{-4} M BrdU, exposure for 24 h, and 20000 cells/well. Incorporated BrdU was detected by an ELISA.

Fig. 5. Changes in BrdU incorporation into isolated rabbit placental cells during pregnancy. The cells (20000/well) were preincubated in DMEM for 24 h and exposed to 10^{-4} M BrdU for another 24 h. The incorporated BrdU was detected by an ELISA. All data represent the mean $\pm$ SD of 4 pregnant rabbits. The difference between all days is significant ($P < 0.01$).

These results, therefore, suggest that rabbit placental growth may be regulated not only by maternal factors but also by local factors.

Purification of cytotrophoblasts from human term placentae by trypsin digestion and Percoll gradient centrifugation has been reported by

Kliman *et al.* [7]. A combination of trypsin and collagenase was used for enzymatic digestion in this study because a small number of cells were obtained from trypsin-digested tissues in our preliminary experiments (data not shown).

It has been demonstrated that cytotrophoblasts specifically react to cytokeratin antibody in the mouse [10], the rhesus monkey [11], and the human [12]. In the rabbit, placental tissue derived from the fetal region, but not maternal, showed positive immunohistochemical staining with a monoclonal cytokeratin antibody (MAK-6). Similarly, the isolated rabbit placental cells had positive staining with MAK-6 and were identified as trophoblastic cells. These results suggest that this method is suitable for purifying placental cells from rabbit placenta.

Measurement of BrdU incorporation into the DNA using BrdU antibody has been useful for assessment of cell proliferation [9, 13]. When day 11 isolated rabbit placental cells were incubated with BrdU, and the incorporated BrdU was detected by means of ELISA, absorbance increased depending exposure time, BrdU concentration, and cell count. Therefore, absorbance appears to adequately reflect the quantitative assessment of cell proliferation in rabbit placental cells.

Specific receptors to growth factors, such as epidermal growth factor (EGF) [14-17], insulin-like growth factors (IGFs) [18, 19], colony-stimulating

factor-1 [20], granulocyte colony-stimulating factor [21], tumor necrosis factor [22], and insulin [23, 24] have been found in the human placenta. These maternal factors have been recognized as regulators of placental growth. There are also several reports suggesting the autocrine control of human

placental growth [25–27]. Although our study suggests the possibility of autonomous regulation of placental growth in the rabbit, the regulatory mechanisms for rabbit placental growth remain to be elucidated.

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