Association of *Dolichos biflorus* lectin binding with full differentiation of bovine trophoblast cells

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Binucleate cells in ruminant placenta are differentiated from fetal mononucleate trophoblast cells and secrete many glycoproteins. This study characterized bovine placental binucleate cells in primary culture and a bovine trophoblastic cell line (BT-1) with an *N*-acetyl-galactosamine-binding lectin, *Dolichos biflorus* agglutinin (DBA). DBA specifically bound to the surface membrane and the cytoplasm of binucleate cells. Mononucleate epithelial cells and fibroblasts were free of DBA. DBA-positive binucleate cells corresponded to the fully matured cells, producing placental lactogen, and the cytoplasm was devoid of cytokeratin. Binucleate cells assumed a flattened shape on a collagen substratum in an extended culture, and entered a dedifferentiated state with a degranulation of placental lactogen. In these flattened cells, DBA reactions were attenuated in the cytoplasm. DBA binding

in BT-1 was subsequently examined. BT-1 was derived from blastocysts produced *in vitro* and is trophectodermal as shown by the expression of cytokeratin. BT-1 was able to differentiate into placental lactogen-producing binucleate cells on a collagen gel substratum. Cytokeratin expression in BT-1 was downregulated with the differentiation into binucleate cells. However, DBA bound to neither mononucleate nor the differentiated binucleate cells in BT-1. These results indicate that binucleate cells *in vivo* but not binucleate cells derived from BT-1, specifically developed glycoconjugates recognized by DBA. The glycoconjugate expression was associated with fully differentiated cells. The onset of DBA binding in binucleate cells coincides with placental development, and binucleate cells differentiated from BT-1 cell cultures may reflect those cells at earlier stages of gestation.

Introduction

Binucleate cells are found in the fetal portion of the placenta in ruminants and primarily serve an endocrine function during pregnancy (for a review, see Wooding and Flint, 1994). These cells produce both peptide- and steroid-hormones, such as placental lactogen (Duello *et al.*, 1986; Wooding and Beckers, 1987; Anthony *et al.*, 1995), prolactin-related protein-1 (PRP-1; Zieler *et al.*, 1990; Kessler *et al.*, 1991; Anthony *et al.*, 1995), pregnancy-associated glycoproteins (PAGs; Zoli *et al.*, 1991, 1992; Green *et al.*, 2000), oestradiol (Matamoros *et al.*, 1994) and progesterone (Reimer *et al.*, 1985; Wango *et al.*, 1991). Binucleate cells are thought to be produced by acytokinesis (Wimsatt, 1951; Björkman, 1968; Klisch *et al.*, 1999b) and by subsequent endoreduplication (Klisch *et al.*, 1999a) of mononuclear trophoblastic epithelial cells, and they represent about 20% of the trophoblastic epithelial cells throughout gestation (Wooding and Wathes, 1980; Wooding, 1982). Binucleate cells show a unique migration with the maintenance of tight junctions between adjacent trophoblastic epithelial cells (Wooding *et al.*, 1994). The migrating cells are destined to fuse with uterine epithelial

cells and secrete hormones into the maternal circulation (Wooding and Wathes, 1980; Wooding, 1982; Wooding and Beckers, 1987). These hormones are thought to influence maternal metabolism (Anthony *et al.*, 1995).

Histochemistry reveals that the granules of binucleate cells are heavily glycosylated as shown by periodic-acid–Schiff staining (Wimsatt, 1951) and by lectin binding (Munson *et al.*, 1989; Lehmann *et al.*, 1992; Jones *et al.*, 1994). Several studies (Munson *et al.*, 1989; Lehmann *et al.*, 1992; Jones *et al.*, 1994) have demonstrated consistently that bovine binucleate cells in placental tissue (but not before day 40 of gestation, Lehmann *et al.*, 1992) stain intensely with *Dolichos biflorus* agglutinin (DBA), a plant lectin with specificity for terminal non-reducing α -linked *N*-acetylgalactosamine glycoresidues (Etzler and Kabat, 1970). The molecular structure and the function of glycoconjugates recognized in bovine binucleate cells by DBA have not been elucidated, but DBA-binding activity provides a useful marker for binucleate cells, and may reflect the state of their differentiation.

Nakano *et al.* (2001) established a primary culture system of binucleate cells from bovine placental tissue and found that fully matured binucleate cells with placental lactogen productivity did not have cytokeratin in the cytoplasm, but immature or dedifferentiated binucleate cells without placental lactogen expressed cytokeratin. In the present study, a detailed analysis of DBA binding was

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Fig. 1. *Dolichos biflorus* agglutinin (DBA) staining of bovine placental binucleate cells in primary culture. After 1 day in culture, binucleate cells were incubated with fluorescein isothiocyanate (FITC)-conjugated DBA in the (b) absence or (d) presence of 0.2 mol *N*-acetylgalactosamine l^{-1} . Nomarski differential images (a) and (c) are the same fields of images as (b) and (d), respectively. Nuclei were stained with Hoechst 33342 and the images were merged into Nomarski differential images. (e–g) FITC-conjugated DBA staining in live

performed on binucleate cells in primary culture and intact tissue in relation to their placental lactogen and cytokeratin expression. Cell cultures allow determination of which types of cell (especially mono- or binucleate cells) are stained with DBA, as the whole appearance of the individual cell can be seen in cell cultures, unlike in tissue sections. In addition, DBA binding on a bovine trophoblastic cell line (BT-1) established from blastocysts produced *in vitro* was examined (Shimada *et al.*, 2001). This cell line is of interest as some cells can transform into placental lactogen-producing binucleate cells when they are cultured on a collagen gel substratum (Nakano *et al.*, 2002).

Materials and Methods

Preparation of bovine placental binucleate cells

Bovine (*Bos taurus*) placentomes were obtained from Holstein cows from mid-gestation to term (fetal crown-rump lengths: 20, 35, 40, 52, 78 and 88 cm) from the local abattoir. Binucleate cells were isolated and cultured as described by Nakano *et al.* (2001). In brief, fetal cotyledonary tissue was separated manually from placentomes, washed three times with PBS (Nissui, Tokyo) and cut into small pieces. The pieces of tissue were incubated with 0.1% (w/v) collagenase (S-1; Nitta Gelatin, Osaka) in Dulbecco's modified Eagle's/F-12 medium (DME/F-12, Gibco, Rockville, MD) containing 100 iu penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (Sigma, St Louis, MO), supplemented with 5% (v/v) fetal bovine serum (Sigma) for 15–25 min at 37°C. The digested tissue was filtered through a series of 300 and 180 µm pore stainless steel meshes to remove undigested tissue. The filtrate was washed with the medium and layered on discontinuous Percoll (Amersham Pharmacia Biotech, Uppsala) gradients with isopycnic densities at 1.03–1.08 g ml⁻¹, and centrifuged at 800 *g* for 20 min. Binucleate cells sedimented at the interface between 1.05 and 1.06 g ml⁻¹ were collected, washed and resuspended in the medium. The cell suspension was plated on collagen-gel mounted coverslips and cultured at 37°C in an atmosphere of 5% (v/v) CO₂.

Culture of BT-1

BT-1 was established from blastocysts produced *in vitro* (Shimada *et al.*, 2001). BT-1 was cultured in DME/F-12 medium containing 100 iu penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹, supplemented with 10% (v/v) fetal bovine serum alone, or with 10% fetal bovine serum, 50% (v/v) bovine uterine fibroblast conditioned medium, 50 µmol β-mercaptoethanol l⁻¹ and 1 µg heparin ml⁻¹. The

uterine fibroblast conditioned medium promoted the growth of BT-1 (Shimada *et al.*, 2001) and was prepared by incubating confluent bovine uterine fibroblasts in DME/F-12 supplemented with 10% fetal bovine serum for 2 or 3 days. The conditioned medium was filtered (0.22 µm) and stored at –20°C until use.

A monolayer of the confluent BT-1 cell sheet was mechanically passaged in and out with a pipette (pore size 2 mm). The dissociated cell clumps in the culture medium were plated on collagen-coated flasks. The cell clumps attached to the flasks and proliferated to form a monolayer again. Successful passages were also achieved by transferring multicellular vesicles formed spontaneously from the cell colony (Shimada *et al.*, 2001). These vesicles (100 µm to 1 mm in diameter) floating in the culture medium were collected with 180 µm pore stainless steel mesh. The vesicles were resuspended in fresh medium and plated on a new collagen-coated flask. The vesicles attached to the collagen substratum, flattened out and regenerated the cell sheet.

The multicellular vesicles were cultured on collagen-gel mounted coverslips in DME/F-12 supplemented with 10% (v/v) fetal bovine serum to induce differentiation into binucleate cells. After > 10 days, clusters of binucleate cells appeared in the cell colony (Nakano *et al.*, 2002).

Preparation of collagen-mounted culture flasks and coverslips

The collagen-coated flasks were prepared by incubating a tenfold diluted solution of acid soluble pig type I collagen (type I-A, 3 mg ml⁻¹, Nitta Gelatin, Osaka) in culture flasks for > 1 h at room temperature (about 26°C). The culture flasks were washed with DME/F-12 and used for cell culture. Collagen-gel mounted coverslips were prepared using the collagen (type I-A) according to the manufacturer's instructions. Eight volumes of the collagen solution was mixed with one volume of a tenfold concentrated Hepes-buffered physiological salt solution (pH 7.4) and one volume of a 0.05 mol NaHCO₃ l⁻¹ buffer solution in a conical tube on ice. A drop of the mixed gel solution was dispensed on to a horizontal glass coverslip (15 or 25 mm, Matsunami) and incubated at 37°C for 20–30 min until use in cell culture.

Lectin and immunocytochemical staining

Cells cultured on collagen-gel mounted coverslips were fixed with 4% (w/v) paraformaldehyde in 0.1 mol phosphate buffer l⁻¹ at 4°C for 15 min. After three washes with PBS, the cells on the coverslips were immersed in

binucleate cells just after isolation. (e) Phase contrast, (f) Hoechst 33342 and (g) FITC-conjugated DBA images in the same field. The surface membrane of a binucleate cell is indicated by an arrow. Binucleate cells were prepared from placentomes at fetal crown-rump lengths of 35 cm (a–d) and 78 cm (e–g). Scale bars represent 30 µm.

Fig. 2. *Dilichos biflorus* agglutinin (DBA) staining of epithelial cells and fibroblasts contaminating binucleate cell preparations. After 1 day in culture, cells were double-stained with (a,c,e) monoclonal anti-cytokeratin antibody (red) and fluorescein isothiocyanate (FITC)-conjugated DBA (green), or (b,d,f) monoclonal anti-vimentin antibody (red) and FITC-conjugated DBA (green). (a,b) Nuclei were stained

acetone at -30°C for 5 min. Lectin staining was performed by incubating $10\text{ }\mu\text{g}$ fluorescein isothiocyanate-conjugated DBA mL^{-1} (FITC-conjugated DBA; Vector, Burlingame, CA) in the reaction solution (PBS containing 1% (w/v) BSA and 0.05% (w/v) NaN_3) for 1 h at room temperature. For absorption control staining, 0.2 mol *N*-acetylgalactosamine L^{-1} (Sigma) was added to the FITC-conjugated DBA solution. Nuclei were stained with Hoechst 33342 (Molecular Probes, Eugene, OR; final concentration $5\text{ }\mu\text{g mL}^{-1}$). After three washes with PBS, the cells were mounted using Perma Fluor, and viewed under an epifluorescence microscope (Zeiss Axobart).

Double staining was performed using FITC-conjugated DBA combined with either anti-cytokeratin, anti-vimentin or anti-placental lactogen antibody. Cells cultured on collagen-gel mounted coverslips were fixed in 4% (w/v) paraformaldehyde and permeabilized with cold acetone as described above. The cells were blocked with 10% (v/v) normal goat serum in PBS for 30 min at room temperature. Incubation with monoclonal mouse anti-cytokeratin antibody (ready to use, AE1/AE3; Nichirei, Tokyo), monoclonal mouse anti-vimentin antibody (1:800, clone V9; DAKO, Glostrup) or monoclonal mouse anti-PL antibody (1:1000 or 1:10 000; Nakano *et al.*, 2001; Takahashi *et al.*, 2001) was carried out for 2 h at room temperature. After three washes with PBS, a mixture of Alexa 546-conjugated goat anti-mouse IgG antibody (Alexa 546 anti-mouse IgG, 1:400; Molecular Probes), FITC-conjugated DBA and Hoechst 33342 in the reaction solution was applied for 1 h at room temperature. After three washes with PBS, the cells were mounted and viewed under the Zeiss Axobart microscope.

Double staining with monoclonal mouse anti-cytokeratin antibody (AE1/AE3) and polyclonal rabbit anti-PL antibody (1:8000) was performed as described by Nakano *et al.* (2001). The signals were visualized using Alexa 546 anti-mouse IgG and Alexa 488-conjugated goat anti-rabbit IgG antibody (Alexa 488 anti-rabbit IgG, 1:200; Molecular Probes).

These staining techniques were also applied to bovine placenta tissue sections with some modifications. Placentomes from two Holstein cows (crown-rump lengths 20 cm and 40 cm) and three Japanese black cows at days 59, 104 and 150 of gestation (crown-rump lengths of 6.5, 21.0 and 39.0 cm, respectively) were frozen. Cryosections ($6\text{--}8\text{ }\mu\text{m}$ in thickness) were cut with a cryostat (HM500, MICROM) and mounted on 3-amino-propyltriethoxysilane-coated glass slides (Matsunami). The sections were fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature and stained as described above, except for incubation of primary antibodies overnight at 4°C .

DBA staining in live binucleate cells

Just after isolation, binucleate cell preparations were incubated with $10\text{ }\mu\text{g}$ FITC-conjugated DBA mL^{-1} and $10\text{ }\mu\text{g}$ Hoechst 33342 mL^{-1} in 5% (v/v) fetal bovine serum DME/F-12 medium for 30 min at room temperature. After the cells were washed with the medium, they were observed under an inverted epifluorescence microscope (IX70, Olympus).

Results

DBA binding in bovine placental binucleate cells in primary culture

A binucleate cell rich fraction (approximately 50% binucleate cells) was obtained by collagenase digestion and Percoll density gradient centrifugation (Nakano *et al.*, 2001). The fraction was plated out on collagen-gel mounted coverslips. After day 1 of culture, binucleate cells were easily recognized by their oval shape ($20\text{--}30\text{ }\mu\text{m}$ in diameter) and two large nuclei (Fig. 1a,c). FITC-conjugated DBA bound to the whole cytoplasm of binucleate cells (Fig. 1b). DBA staining was attenuated in the presence of its competing sugar, *N*-acetylgalactosamine (Fig. 1d), indicating that the binding was specific. When DBA was applied to live binucleate cells just after isolation (Fig. 1e–g), DBA bound to the surface membrane of binucleate cells (Fig. 1g). Staining of DBA combined with either anti-cytokeratin or anti-vimentin was used to assess the binding to mononucleate epithelial cells and fibroblasts in the binucleate cell preparations. Mononucleate epithelial cells (Fig. 2a,c,e) and fibroblasts (Fig. 2b,d,f) were marked selectively by anti-cytokeratin (Fig. 2c) and anti-vimentin (Fig. 2d), respectively. Almost all ($>95\%$) of the epithelial cells (Fig. 2e) and all of the fibroblasts (Fig. 2f) were free of DBA, whereas binucleate cells were stained strongly (Fig. 2e,f). These results provide clear evidence that DBA specifically labels binucleate cells in primary culture.

Binucleate cells are thought to be produced by the cytokinesis of mononucleate epithelial cells (Wimsatt, 1951; Björkman, 1968; Klisch *et al.*, 1999b), and mature to develop secretory machinery for hormone production. Nakano *et al.* (2001) demonstrated that the cytoplasm of fully matured binucleate cells with placental lactogen productivity did not contain cytokeratin, but that the immature or dedifferentiated cells without placental lactogen expressed cytokeratin. Double staining of DBA and placental lactogen in binucleate cells prepared from three different pregnancies was performed to examine the relationship between DBA binding activity and the maturity of the cell. After day 1 of culture, most ($>80\%$) of the binucleate cells with an oval shape were placental

with Hoechst 33342. (a,c,e) Mononucleate epithelial cells (arrows) and (b,d,f) fibroblasts (arrowheads) are marked by (c) anti-cytokeratin and (d) anti-vimentin, respectively. (a–f) Binucleate cells are indicated by asterisks. Binucleate cells were prepared from placentomes at crown-rump lengths of 35 cm (a,c,e) and 20 cm (b,d,f). Scale bars represent $30\text{ }\mu\text{m}$.

Fig. 3. Double staining for *Dolichos biflorus* agglutinin (DBA) and placental lactogen (PL) in binucleate cells in primary culture. After 1 day in culture, binucleate cells were double-stained with (b) monoclonal anti-PL antibody (red) and (c) fluorescein isothiocyanate (FITC)-conjugated DBA (green). (a) Nuclei were stained with Hoechst 33342 and the image was merged into the Nomarski differential image. Binucleate cells were prepared from placentomes at a crown–rump length of 52 cm. Scale bar represents 30 μ m.

Table 1. Frequency of bovine placental binucleate cells labelled with *Dolichos biflorus* agglutinin (DBA) and placental lactogen (PL)

	PL(+)/DBA(+)	PL(+)/DBA(–)	PL(–)/DBA(+)	PL(–)/DBA(–)	Total number
Sample 1	119 (98.4)	0 (0.0)	1 (0.8)	1 (0.8)	121 (100.0)
Sample 2	124 (82.7)	0 (0.0)	21 (14.0)	5 (3.3)	150 (100.0)
Sample 3	75 (74.3)	6 (5.9)	10 (9.9)	10 (9.9)	101 (100.0)

Binucleate cells from three different pregnancies (fetal crown–rump lengths: sample 1 = 20 cm, sample 2 = 52 cm, sample 3 = 88 cm) were double-stained with DBA and PL after 1 day in culture. Binucleate cells of an oval shape were counted to classify PL-positive and DBA-positive (PL(+)/DBA(+)), PL-positive and DBA-negative (PL(+)/DBA(–)), PL-negative and DBA-positive (PL(–)/DBA(+)), and PL-negative and DBA-negative (PL(–)/DBA(–)) populations. Numbers in parentheses denote the percentage.

Table 2. Frequency of bovine placental binucleate cells labelled with *Dolichos biflorus* agglutinin (DBA) and cytokeratin (CK)

	CK(+)/DBA(+)	CK(+)/DBA(–)	CK(–)/DBA(+)	CK(–)/DBA(–)	Total number
Sample 1	6 (4.7)	2 (1.6)	119 (92.9)	1 (0.8)	128 (100.0)
Sample 2	10 (9.3)	4 (3.7)	93 (87.0)	0 (0.0)	107 (100.0)
Sample 3	23 (13.4)	21 (12.2)	126 (73.2)	2 (1.2)	172 (100.0)

Binucleate cells from three different pregnancies (fetal crown–rump lengths: sample 1 = 20 cm, sample 2 = 52 cm, sample 3 = 88 cm) were double-stained with DBA and CK after one day in culture. Binucleate cells of an oval shape were counted to classify CK-positive and DBA-positive (CK(+)/DBA(+)), CK-positive and DBA-negative (CK(+)/DBA(–)), CK-negative and DBA-positive (CK(–)/DBA(+)), and CK-negative and DBA-negative (CK(–)/DBA(–)) populations. Numbers in parentheses denote the percentage.

lactogen-positive (Fig. 3b), and almost all of these cells were co-stained with DBA (Fig. 3c). Similar observations were obtained from three different preparations (Table 1). Some placental lactogen-negative binucleate cells were stained

by DBA, but such populations were small and variable between different preparations (0.8–14.0% of total cells, Table 1). In these cells, DBA binding was localized at the surface membrane and the cytoplasm, but in some cells

Fig. 4. *Dolichos biflorus* agglutinin (DBA) staining of flattened binucleate cells. Double staining with (a) monoclonal anti-placental lactogen (PL) antibody (red) and (b) fluorescein isothiocyanate (FITC)-conjugated DBA (green) was performed in binucleate cell of a flattened shape after 1 day in culture. (c) DBA staining of flattened binucleate cell after 3 days in culture. Binucleate cells were prepared from placentomes at a crown-rump length of 40 cm. Scale bars represent 30 μ m.

only to the surface membrane. Double staining for DBA and cytokeratin indicated that DBA-positive signals were mainly detected in the binucleate cells without cytokeratin (Table 2). These results indicated that the DBA binding activity was associated with the state of differentiation of the binucleate cell; DBA predominantly recognized fully matured binucleate cells with placental lactogen-positive and cytokeratin-negative signals.

Some binucleate cells had extended on the collagen substratum after day 1 of culture, and assumed a flattened shape (Fig. 4a,b). In these cells the expression of placental lactogen was downregulated (Fig. 4a). The cytoplasmic staining of DBA was also attenuated with a change in shape, and the DBA signal was localized to the peri-nuclear region (Fig. 4b). After day 4 in culture, most binucleate cells had assumed a flattened shape with a downregulated expression

Fig. 5. Bovine placenta tissue stained with placental lactogen (PL), *Dolichos biflorus* agglutinin (DBA) and cytokeratin. Tissue sections of placentome at day 59 of gestation were double-stained with (a,c,e) polyclonal anti-PL (green) and monoclonal anti-cytokeratin antibodies (red) or with (b,d,f) fluorescein isothiocyanate (FITC)-conjugated DBA (green) and the anti-cytokeratin antibody (red). (a,b) Nuclei were stained with Hoechst 33342. (a–f) A binucleate cell in chorionic villi (CV) is indicated by an arrow. (a,c,e) Uterine epithelial cell layers in caruncular septa (CS) are indicated by arrowheads. Scale bars represent 50 μm.

Fig. 6. Double staining for placental lactogen (PL) and cytokeratin in binucleate cells differentiated from the bovine trophoblast cell line (BT-1). After 15 days in collagen gel culture, BT-1 cells were double-stained with (b) polyclonal anti-PL and (c) monoclonal anti-cytokeratin antibodies. (a) Nuclei were stained with Hoechst 33342. Some differentiated binucleate cells are indicated by arrowheads. (a–c) A cluster of binucleate cells formed on the cell sheet is indicated by an asterisk. Scale bar represents 50 μm .

of placental lactogen (Nakano *et al.*, 2001). The DBA signal was further attenuated, and appeared sparsely throughout the cytoplasm (Fig. 4c). The results indicated that a decrease in DBA binding activity and placental lactogen immunoreactivity in binucleate cells coincided with a flattened shape in a dedifferentiated state.

DBA binding in bovine placental tissue

Tissue sections of bovine placentome were examined at day 59 of gestation, as DBA binding to binucleate cells has been observed at day 40 of gestation (Lehmann *et al.*, 1992). After double staining of anti-placental lactogen and anti-cytokeratin (Fig. 5a,c,e), binucleate cells labelled with anti-placental lactogen (Fig. 5c) were devoid of anti-cytokeratin staining (Fig. 5e), whereas adjacent mononucleate trophoblast cells in chorionic villi were stained intensely with anti-cytokeratin. Reduced staining of anti-cytokeratin was observed in the uterine epithelial cell layer of caruncular septa (Fig. 5e). DBA staining combined with either anti-placental lactogen or anti-cytokeratin (Fig. 5b,d,f) showed that the cytoplasm of binucleate cells (but not mononucleate trophoblast cells) in chorionic villi was stained intensely with DBA (Fig. 5d), and that these cells had placental lactogen-positive (data not shown) and cytokeratin-negative signals (Fig. 5f). Uterine epithelial cells in caruncular septa were also stained somewhat with DBA (Fig. 5d). Similar observations were obtained with placentomes at later stages of gestation (approximately day 100 and day 150), except that there was more intense staining of DBA at the basal region of uterine epithelial cells in caruncular septa (data not shown). These results confirm the findings of the present study in primary cell cultures in which DBA bound to binucleate cells with placental lactogen-positive and cytokeratin-negative signals.

DBA binding in BT-1

BT-1 is a bovine trophoblastic cell line derived from blastocysts produced *in vitro*, and its trophectodermal properties are characterized by the expression of cytokeratin (Shimada *et al.*, 2001; Nakano *et al.*, 2002). Some BT-1 cells (2–3%) were able to differentiate into binucleate cells when cultured on a collagen gel substratum (Nakano *et al.*, 2002) (Fig. 6). These binucleate cells were often observed in the stratified area of the cell colony (Fig. 6). The binucleate cells had an increased DNA content presumably due to endoreduplication (Nakano *et al.*, 2002), and many of the cells produced placental lactogen (Nakano *et al.*, 2002) (Fig. 6b). In the present study, the expression of cytokeratin filaments in the binucleate cells was often downregulated in the cytoplasm like those *in vivo* (Fig. 6c). Double staining of DBA and placental lactogen revealed that DBA did not bind to either the mononucleate or the differentiated binucleate cells irrespective of placental lactogen immunoreactivity (Fig. 7). These results indicate that binucleate cells in BT-1 were similar to those cells *in*

Fig. 7. Double staining for placental lactogen (PL) and *Dolichos biflorus* agglutinin (DBA) in binucleate cells differentiated from the bovine trophoblast cell line (BT-1). After 17 days in collagen gel culture, BT-1 cells were double-stained with (b) monoclonal anti-

in vivo in terms of placental lactogen and cytokeratin expression, but were distinct in DBA staining.

Discussion

The present study characterized bovine placental binucleate cells in primary culture and a bovine trophoblastic cell line (BT-1) with a *N*-acetylgalactosamine-binding lectin, DBA. DBA specifically recognizes a terminal non-reducing α -linked *N*-acetylgalactosamine residue in glycoconjugates (Etzler and Kabat, 1970). The present study demonstrated that DBA selectively binds to binucleate cells (not to mononucleate epithelial cells or fibroblasts) isolated from the placenta at mid-gestation to near term. BT-1 is a trophoblastic cell line with the ability to differentiate into binucleate cells (Shimada *et al.*, 2001; Nakano *et al.*, 2002). However, DBA did not bind to either the mononucleate or the differentiated binucleate cells in BT-1. These results indicate that binucleate cells *in vivo*, but not those in BT-1, produced DBA-recognized glycoconjugates.

The results from the present study that DBA binds specifically to binucleate cells confirmed findings obtained using bovine placental tissue sections (Munson *et al.*, 1989; Lehmann *et al.*, 1992; Jones *et al.*, 1994). The DBA binding to binucleate cells in bovine placental tissue occurs between day 29 and day 40 of gestation (Lehmann *et al.*, 1992), and is maintained until near term (Munson *et al.*, 1989; Jones *et al.*, 1994). Binucleate cells contain a well-developed rough endoplasmic reticulum and Golgi apparatus (Björkman, 1968), and produce many glycoproteins including placental lactogen (Shimomura and Bremel, 1988), PRP-1 (Zieler *et al.*, 1990), PAGs (Zoli *et al.*, 1991; Green *et al.*, 2000) and the SBU-3 antigen of chorionic villi (Lee *et al.*, 1986). Therefore, it is conceivable that a lectin such as DBA can bind to glycans expressed by binucleate cells. In the present study, it was demonstrated that DBA-positive signals largely corresponded to binucleate cells with placental lactogen-positive and cytokeratin-negative signals in primary culture and also in intact tissue. Binucleate cells are thought to be produced by the acytokinesis of cytokeratin-positive epithelial cells in the trophoblast layer (Wimsatt, 1951; Björkman, 1968; Klisch *et al.*, 1999b), and then develop secretory machinery for hormone production (Wooding, 1982) with a concomitant decrease in cytokeratin expression (Lee *et al.*, 1990; Nakano *et al.*, 2001). The results indicate that in addition to placental lactogen and cytokeratin expression, DBA binding activity also reflects the maturity of binucleate cells.

DBA binding was recognized at the surface membrane and the cytoplasm of binucleate cells. In the cytoplasm,

PL (red) and (c) fluorescein isothiocyanate (FITC)-conjugated DBA (green). (a) Nuclei were stained by Hoechst 33342. Note both PL-positive (arrowheads) and PL-negative (arrows) binucleate cells are free from DBA staining. Scale bar represents 50 μ m.

DBA binding would be localized in granules of secretory vesicles, as the DBA binding was correlated with hormone synthesis of the cells marked by placental lactogen. In some placental lactogen-negative binucleate cells, DBA stained only the surface membrane of the cells. However, DBA did not seem to bind to placental lactogen because terminal positions of glycosidues in placental lactogen are expected to be occupied by sialic acid residues (Shimomura and Bremel, 1988). The cytoplasmic staining of DBA in some placental lactogen-negative binucleate cells may be explained by the presence of heterogeneous population in hormone synthesis (Wooding *et al.*, 1996).

Binucleate cells in culture underwent a change in shape from oval to flat within a few days, and entered a dedifferentiated state with a downregulated expression of placental lactogen (Nakano *et al.*, 2001). Thus, the decreased staining of DBA in the cytoplasm of flattened binucleate cells may result from the depletion of glycosylated secretory vesicles. The peri-nuclear region recognized by DBA in flattened binucleate cells would be the Golgi apparatus in which the glycosylation of proteins predominantly occurs.

The binucleate cells differentiated from BT-1 were free from DBA binding in contrast to the binucleate cells in primary culture and tissue, although the cells differentiated from BT-1 have similar properties to the cells in primary culture in respect of placental lactogen production and an increased nuclear DNA content (Duello *et al.*, 1986; Klisch *et al.*, 1999a; Nakano *et al.*, 2002). The present study demonstrated that the expression of cytokeratin filaments of binucleate cells in BT-1 was also downregulated. Thus, *in vivo*- and *in vitro*-derived binucleate cells were similar except for DBA binding activity. In bovine placenta, DBA-positive binucleate cells are first recognized between day 29 and day 40 of gestation (Lehmann *et al.*, 1992), although binucleate cells are already present and have secreted placental lactogen at day 17 (Flint *et al.*, 1979). BT-1 was derived from bovine blastocysts fertilized *in vitro*, and retained trophectodermal properties with the expression of cytokeratin and interferon- τ (Shimada *et al.*, 2001; Nakano *et al.*, 2002). Interferon- τ is an anti-luteolytic factor, largely secreted from mononucleate cells of the trophectoderm at peri-implantation (day 17 of gestation in cows, Demmers *et al.*, 2001). Thus, binucleate cells differentiated from BT-1 cell cultures may reflect those cells at the early stages of gestation.

The function of glycoconjugates recognized by DBA in binucleate cells remains to be elucidated. Another glycoprotein, the SBU-3 antigen, which is found in secretory granules and Golgi apparatus in bovine binucleate cells, is also not detected at an early stage (day 18–23), but is detected at day 30 of gestation (Morgan *et al.*, 1989). The appearance of the SBU-3 antigen coincides with the development of the chorionic villi, implying its involvement in placentation (Morgan *et al.*, 1989). In particular, binucleate cells show cell migration and cell fusion at the interface between the chorionic villi and maternal uterine

caruncle (Wooding and Wathes, 1980; Wooding, 1982; Wooding and Beckers, 1987; Wooding *et al.*, 1994). These glycoconjugates in granules and on the cell surface may have a role in cell recognition and cell-to-cell interaction. Further study will be needed to clarify which glycoconjugates are recognized by DBA, and also their relationship with cellular function.

In conclusion, DBA selectively binds to matured binucleate cells with placental lactogen-positive and cytokeratin-negative signals in primary culture and tissue, but not to those cells in BT-1. Thus, DBA-binding activity represents the full differentiation into binucleate cells *in vivo*. The onset of DBA binding in binucleate cells coincides with placentation (at about day 30 of gestation), and binucleate cells differentiated from BT-1 cell cultures may represent those cells at earlier gestation.

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