The glycosylation of pregnancy-associated glycoproteins and prolactin-related protein-I in bovine binucleate trophoblast giant cells changes before parturition

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Abstract

Binucleate trophoblast giant cells (BNC) in the bovine placenta produce glycoproteins, which are delivered into the mother after fusion of BNC with uterine epithelial cells. During most time of pregnancy, BNC produce pregnancy-associated glycoproteins (PAGs) and prolactin-related protein-I (PRP-I) with asparagine-linked lactosamine-type glycans terminating with *N*-acetyl-galactosamine. We show by lectin histochemistry that terminal *N*-acetyl-galactosamine (detected by *Dolichos biflorus* agglutinin, DBA) in placental BNC is greatly reduced prior to parturition, while lactosamine-type *N*-glycans (detected by *Phaseolus vulgaris* leucoagglutinin, PHA-L) remain unaltered. The change in DBA-staining showed no statistically significant differences between placentomes of cows with and without retention of fetal membranes. Western blots revealed that, at parturition the apparent molecular mass of PAGs and PRP-I is 1–2 kDa lower than in late pregnancy. These changes are due to alterations of asparagine-linked glycans, since the molecular weight of the peptide backbones after enzymatical release of asparagine-linked glycans is identical at late pregnancy and parturition. Lectin western blots showed a reduction of terminal *N*-acetyl-galactosamine on PAGs at parturition. A lectin sandwich-ELISA was used to differentiate DBA- and PHA-L-binding PAGs in sera of pregnant and non-pregnant cows. The values for DBA-binding PAGs at parturition were not significantly different from non-pregnancy, while the values for PHA-L-binding PAGs were significantly higher at parturition. The peripartal changes of PAG- and PRP-I-glycosylation could alter functional properties of these proteins and might therefore be considered for functional studies. The differentiation of PAG glycoforms in maternal serum could be valuable for a further optimization of PAG-based pregnancy diagnosis in cattle.

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Introduction

Binucleate trophoblast giant cells (BNC) are a characteristic feature of ruminant placenta (Wimsatt 1951, Wooding 1992). The BNC develop from uninucleate trophoblast cells and become binucleate by acytokinetic mitosis (Wimsatt 1951, Wooding 1992, Klisch *et al.* 1999a). During cell maturation, BNC become polyploid (Klisch *et al.* 1999b) and large amounts of dense core vesicles accumulate in their cytoplasm. Mature BNC migrate towards the uterine epithelium and fuse with uterine epithelial cells (Wooding 1992). This results in

the formation of trinucleate feto-maternal hybrid cells, which release their granules at the basal membrane of the uterine epithelium. Therefore, the BNC facilitate the transport of trophoblast-derived proteins across the otherwise protein-tight synepitheliochorial placental barrier (Wooding & Flint 1994).

The BNC granules bind some lectins (*Phaseolus vulgaris* leucoagglutinin, PHA-L; *Dolichos biflorus* agglutinin, DBA; *Vicia villosa* agglutinin, VVA) with high specificity (Munson *et al.* 1989, Jones *et al.* 1994, Nakano *et al.* 2002, Klisch & Leiser 2003). These lectins

recognize terminal *N*-acetylgalactosamine (DBA, VVA; Piller *et al.* 1990) or an oligosaccharide motif in complex asparagine-linked glycans of the lactosamine-type (PHA-L; Hammarström *et al.* 1982, Green & Baenziger 1987, Kaneda *et al.* 2002). It has been demonstrated that in bovine BNC granules, PAGs and PRP-I are the main glycoproteins to which these lectins bind (Klisch & Leiser 2003, Klisch *et al.* 2005).

PAGs of ruminants form a large protein family (Green *et al.* 1998). In cattle, 21 PAG mRNAs have been sequenced (Green *et al.* 2000), but the number of PAG genes in the bovine and ovine genome has been estimated to be at least 100 (Xie *et al.* 1997). PAGs belong to the protein family of aspartic proteinases (Xie *et al.* 1991), but to date no proteolytic activity of ruminant PAGs has been demonstrated. Due to the transfer of PAGs from the trophoblast to the maternal placental stroma and vasculature, the proteins can be detected in the maternal blood during pregnancy and therefore gained importance for pregnancy diagnosis in ruminants (Sasser & Ruder 1987, Zoli *et al.* 1992, Green *et al.* 2005).

PRP-I belongs to a gene family which, like ruminant placental lactogen, arose from the prolactin gene by gene duplication(s; Forsyth & Wallis 2002). Several different PRP-mRNAs have been identified (Ushizawa *et al.* 2005), but PRP-I has been demonstrated on the protein level only (Kessler & Schuler 1997, Klisch *et al.* 2005). No binding of PRP-I to prolactin or growth hormone receptors has been demonstrated, and to date PRP-I has not been detected in the maternal circulation (Kessler & Schuler 1997).

In the present study, we demonstrate by lectin histochemistry and lectin western blot that the glycosylation of PAGs and PRP-I in bovine BNC changes before parturition. Similar changes of glycosylation are detected by a lectin-ELISA on PAGs in the serum of late pregnant cows.

Materials and Methods

Animals

Placentomal tissue for histochemical procedures was obtained from three groups of cows.

- I Midpregnancy placentas ($n=5$; days 95, 110, 115, 170, and 200) were obtained from a local slaughterhouse. The fetal ages were estimated from the crown-rump length (Schnorr & Kressin 2001).
- II Term placentas ($n=4$) were obtained from cows undergoing routine cesarean section for fetal/pelvic mismatch after spontaneous onset of birth. Spontaneous onset of birth means spontaneous opening of the cervix, onset of labor, and rupture of fetal membranes after physiological gestational length. The cow was allowed to continue with labor until dystocia became obvious for the respective owner

(between 1 and 3 h after the onset of labor). The cows were then taken to the clinic, where the cesarean sections were performed immediately. No treatment was performed to induce parturition. Placentomes were collected immediately after delivery of the normally developed calves.

- III Single placentomes were obtained from 11 cows within 1 h after spontaneous calving. Whole placentomes were removed from the uterus with an effinator after Reisinger, modified by Richter (Hauptner, Solingen, Germany), which is a clamping, crushing, and cutting instrument, originally constructed for the removal of ovaries from large animals. This instrument was used to prevent any bleeding from the caruncular stalk into the uterine lumen after removal of a placentome. In the following, the cows were examined for the release of fetal membranes. In six cows, the fetal membranes were released within 12 h after expulsion of the fetuses (REL) and five animals retained the fetal membranes for more than 12 h (RET).

For western analysis, placentomal tissue, consisting of unseparated cotyledon and caruncle, was obtained from a local slaughterhouse (day 240, $n=1$; day 260, $n=2$). Similar tissue from three term pregnancies was obtained by cesarean sections at spontaneous birth, which were carried out at the clinic of veterinary obstetrics (University of Giessen, Germany).

Blood samples for the lectin-ELISA were collected at the experimental farm and the clinic for cattle of the veterinary school, Hannover, Germany. Samples were taken from cows at day 210 ($n=5$), day 260 ($n=5$), partus ($n=7$), and from non-pregnant cows at least 100 days *post partum* ($n=10$). Animal experiments were approved by the competent authorities of Hannover and Giessen districts, and were performed according to the German Law for the protection of Animals (TierschG).

Lectin histochemistry

The placentomes were immediately cut into slices (5 mm thick), which extend from the surface to the base of the placentome. These slices were fixed in 4% formaldehyde (v/v) in 0.1 M phosphate buffer (pH 7.3) for 24 h and triangular pieces, which include all strata of the placentome, were embedded in paraffin. Sections (7 μ m) were dewaxed in xylol, rinsed in three changes of ethanol, rehydrated in descending concentrations of ethanol, and rinsed in distilled water. The slides were rinsed in 0.05 M Tris-buffered saline (pH 7.6), 1 mM CaCl_2 (TBS), and incubated for 45 min in a humid chamber at 37 °C with 10 μ g/ml biotinylated lectin (DBA; Sigma; PHA-L, EY-Laboratories, San Mateo, CA, USA) in TBS. After washing twice in TBS, the slides were incubated with a preformed streptavidin/biotin-peroxidase (ABC) complex for 60 min at 37 °C, rinsed in PBS, and

developed in PBS containing 0.02% (w/v) diaminobenzidine (DAB), 0.3% (w/v) ammonium nickel (III)-sulfate, and 0.015% (v/v) H₂O₂.

As controls, the lectins were replaced by TBS or 0.2 M *N*-acetylgalactosamine (GalNAc) was added to the buffer during incubation with DBA.

Immunohistochemistry

Sections were dewaxed and rehydrated as described previously. After this, the slides were washed in PBS for 2 min, blocked with 5% (w/v) BSA in PBS for 30 min and incubated with polyclonal rabbit antisera in a moist chamber overnight. Anti-PAG (PAG-F4, a gift from Drs J Green and R M Roberts, St Louis, Missouri, USA) was diluted in the ratio of 1:2000 in PBS with 1% BSA, and anti-bovine PRP-I (Zieler *et al.* 1990; a gift from Dr L A Schuler, Department of Comparative Biosciences, University of Wisconsin-Madison, Madison, WI, USA) was diluted in the ratio of 1:2000–1:4000 in PBS. Slides were washed twice in PBS and incubated for 30 min with biotinylated anti-rabbit-IgG (1:100) in PBS. After washing twice in PBS, the slides were incubated with a preformed streptavidin/biotin-peroxidase (ABC) complex, washed in PBS, developed in DAB/ammonium nickel(III) sulfate-solution as above, and coverslipped. In controls, the antisera were replaced by buffer or by diluted (1:2000) normal Rabbit Immunoglobulin Fraction (DAKO, Hamburg, Germany).

Western analysis

Slices, expanding from the surface to the base of placentomes, were homogenized in ten volumes Hepes buffer (0.01 M Hepes and 0.15 M NaCl), homogenates were centrifuged (15 000 *g*; 30 min), and the pellets were discarded. The supernatants were stored at –80 °C until analysis. Enzymatical release of *N*-glycans was performed with PNGase F (New England Biolabs, Frankfurt, Germany) according to the manufacturer's instructions. Briefly, the samples were denatured in denaturation buffer (100 °C; 10 min) and the recommended quantities of NP-40 and reaction buffer were added. Finally, 500 U PNGase F per 20 µg protein were added and the mixture was incubated at 37 °C for 3 h. In the control samples, reaction buffer was added instead of PNGase F. The samples (10 µg protein (Bradford assay)) were separated by SDS-PAGE on 12% gels and transferred to PVDF membranes. For staining with antisera (anti-PAG-F4; anti-PRP-I), the membranes were blocked with 6% (w/v) non-fat-dried milk in PBS and incubated with the antisera (1:1000) in PBS (containing 1% BSA for the PAG-F4 antiserum) for 1 h. Blots were washed thrice for 10 min in non-fat-dried milk/PBS, incubated with biotinylated anti-rabbit IgG (1:400) for 45 min, washed and incubated in streptavidin

peroxidase for 45 min. Bound antisera were visualized with ECL (ECL Plus Western Blotting Detection Reagents; Amersham Biosciences, Freiburg, Germany). The blots were stripped with Re-Blot Western Blot recycling kit (Chemicon, Schwalbach, Germany) and reused for lectin staining. Therefore, the membranes were blocked with 2% BSA, 0.5% Tween 20 in TBS for at least 1 h, incubated with 1 µg/ml lectin (PHA-L; DBA) in TBS for 1 h, washed thrice with TBS, and incubated for 1 h in 0.05 µg/ml peroxidase-conjugated streptavidin (Dianova, Hamburg, Germany) in TBS. After washing thrice in TBS, the bound lectins were visualized with ECL as described previously.

Lectin-ELISA

All serum samples were stored at –20 °C until analysis. The samples were measured in duplicates and all incubations were carried out at room temperature. One hundred microliters rabbit anti-PAG serum (R727, from the Laboratory of JF Beckers, University of Liège, Liège, Belgium) diluted in the ratio of 1:4000 in PBS were mixed with 100 µl bovine serum and was incubated on a shaker for 2 h. Biotinylated lectin (0.5 µg PHA-I or DBA in 10 µl PBS) was added, incubated for further 2 h, and then the mixture was transferred into a streptavidin-coated microtiter plate (BioBind; Thermo, Dreieich, Germany) and incubated overnight on a shaker. After washing thrice with 300 µl PBS, 150 µl peroxidase-linked anti-rabbit IgG (Amersham Biosciences; 1:2000 in PBS) was added and incubated for 2 h. The wells were washed thrice with PBS, and 100 µl ABTS (2,2'-azino-di(3-ethyl benthiazoline sulfonic acid) solution (0.05% (w/v) in 50 mM citric acid (pH 4) with 0.03% H₂O₂) was added. After 30 min, the reaction was stopped with 100 µl 1% SDS and read on an Elx800 Microplate reader (Biotec, Winooski, Vermont, USA) at 405 nm.

Results

Lectin and immunohistochemistry

During midpregnancy (group I), the BNC granules were stained with PAG and PRP-I antisera, and with both lectins (DBA, PHA-L; Fig. 1). In addition, the PRP-I antiserum showed a moderate staining of the maternal stroma in all the three groups. In the term placentas (group II), both antisera bound to BNC, which were reduced in number. All remaining BNC at this stage of pregnancy stained with PHA-L, but DBA-positive BNC were reduced considerably and completely absent occasionally (Fig. 1). In some cases, the DBA-positive BNC were clustered in fetal villi, which were surrounded by the otherwise negative tissue (Fig. 2). Controls were consistently negative (Fig. 3).

In group III, the number of lectin-stained BNC was counted in ten microscopical fields of vision (each

Figure 1 Representative serial sections of midpregnant (day 180) and term placentomes. BNC are stained with the antisera (PAG, PRP-I) and PHA-L lectin. DBA binds to the BNC at midpregnancy, but the specific staining is absent at term. Scale bar represents 200 μm .

0.75 mm²). There was no significant difference (Mann–Whitney rank sum test) between animals with retention of fetal membranes (RET) and those which released fetal membranes within 12 h *post partum* (REL; Fig. 4).

Western analysis

In the late pregnancy samples, the major glycosylated PAG isoform had an estimated molecular weight (MW) of approximately 66 kDa, with minor isoforms at 56 and 75 kDa (Fig. 5A). The MW of PAGs was reduced to

Figure 2 In term placenta, PHA-L binds to several BNC which are diffusely distributed. DBA binds only to a subpopulation of BNC, which are located with a high cell density in a single fetal villus. Scale bar represents 200 μm .

Figure 3 Immunostaining of term placenta with the PAG-F4 antiserum and a normal rabbit immunoglobulin fraction as control. Scale bar represents 200 μ m.

37 kDa by PNGase-F treatment. In the term samples, the expression of PAGs was strongly reduced and the main band was observed at 64 kDa.

The expression of PRP did not change obviously between late pregnancy and term, but the glycosylated form in late pregnancy showed a slightly higher apparent MW (36 kDa), compared with term (35 kDa; Fig. 5B). The antiserum also bound to glycoproteins of 44 kDa (late pregnancy) or 42.5 kDa (term).

In late pregnancy, PHA binds to the three bands of PAG (75, 66, and 56 kDa), to PRP (36 kDa), and to the 44 kDa glycoprotein (Fig. 5C). At term, the binding to the 44 kDa glycoprotein and PRP remains relatively strong. In term placenta samples, faint bands at 64 kDa can be seen.

The binding of DBA to PAGs in late pregnancy is clearly visible, but, compared with PHA-L, the binding to PRP is much less intensive (Fig. 5D). At term, no binding to PAG or PRP can be observed. There is only binding to two non-N-glycosylated proteins of 75 and > 100 kDa.

Lectin-ELISA

When biotinylated PHA-L was used to immobilize PAGs on the streptavidin-coated microtiter plates, the measured optical densities (OD) of non-pregnant cows (OD 0.20; ± 0.016) were significantly lower ($P < 0.05$; Dunn's multiple comparison test) than day 210 (1.55; ± 0.49), day 260 (1.30; ± 0.55), and term samples (1.36; ± 0.48 ; Fig. 6A).

The OD values for DBA-binding PAG (Fig. 6B) tended to decrease steadily between day 210 (1.93; ± 0.92), day 260 (1.05; ± 0.92), and term (0.59; ± 0.46), but differences were not significant ($P > 0.05$). Term and non-pregnant samples (0.32; ± 0.04) did not differ significantly, while the latter were significantly lower than values obtained at 210 and 260 days ($P < 0.05$).

Discussion

Glycosylation is a major post-translational modification of proteins, which influences several protein characteristics like receptor binding, intracellular sorting, and resistance to enzymatical degradation (Gabius *et al.* 2004, Sinclair & Elliott 2005). The glycosylation

machinery of ruminant BNC attaches unusual glycans to their major secretory proteins. The glycosylation pattern has been investigated in several lectin histochemical studies (Munson *et al.* 1989, Lehmann *et al.* 1992, Jones *et al.* 1994, Nakano *et al.* 2002, Klisch & Leiser 2003). The binding of PHA-L indicates the presence of N-acetylglucosamine-type glycans (Hammarström *et al.* 1982, Green & Baenziger 1987, Kaneda *et al.* 2002). This kind of glycosylation appears to be conserved within the bovinds, since PHA-L also binds to granules of ovine (Jones *et al.* 1994) and water buffalo (Carvalho *et al.* 2006) BNC. In sheep, it has been demonstrated that PHA binds to PAG, which was immunopurified with the SBU-3 antibody (Atkinson *et al.* 1993). The present study shows that in cattle the PHA-L-binding epitope is constantly expressed around parturition, since BNC at parturition are still stained with PHA-L. The present results of the western analysis confirm earlier findings that PHA-L binds to PAGs and PRP-I (Klisch & Leiser 2003).

A specific feature is the binding of DBA to BNC granules, which occurs in early pregnancy between days 29 and 40 (Lehmann *et al.* 1992) and continues close to term (day 270; Jones *et al.* 1994). Recently, it was shown that GalNAc is part of N-glycans of bovine PAGs and

Figure 4 Number of DBA and PHA-L positive cells in ten microscopical fields (each 0.75 mm²). Data are expressed as mean $\pm$ s.d. No significant difference between animals with a retention of fetal membranes (RET, $n=5$) and those which released fetal membranes within 12 h (REL, $n=6$) were observed.

PRP-I (Klisch & Leiser 2003, Klisch *et al.* 2005). The strongly reduced binding of DBA to BNC in the term placenta demonstrates the absence of terminal GalNAc in BNC at parturition. One explanation for the absence of lectin binding could be that GalNAc is masked by the addition of other molecules to the glycans. For example, the addition of sulfate to GalNAc inhibits the binding of VVA (Green *et al.* 1987). A second

explanation could be that no GalNAc is attached to N-glycans around parturition. The latter possibility appears more likely, since the reduced MW of periparturent PAGs (and PRP-I) could simply result from the absence of GalNAc. Possibly, the GalNAc-transferase, which adds GalNAc to glycans on PAGs, could be regulated by the prepartal shift in placental steroidogenesis from the predominant production of progesterone and biologically inactive conjugated estrogens to active free estrogens (Schuler *et al.* 1994, Hoffmann *et al.* 1997). A downregulation by estrogens has been described for the glycoenzyme GalNAc-transferase (Dharmesh & Baenziger 1993).

The highly specific glycosylation pattern of BNC-derived secretory proteins suggests that the glycans might have specific functions. The specific glycosylation of PAGs might be important for immunoregulatory functions during pregnancy. Recently, it was shown that PAGs produced by BNC are deposited in the stromal layer of the caruncles (Wooding *et al.* 2005). At this location, PAG-glycans might interact with selectins, and could thereby inhibit selectin-mediated cell adhesion. This could be important for the absence of leucocytes in the bovine placental tissue (Lee *et al.* 1997). The role of the observed changes of glycosylation is still completely obscure, but the fact that there are changes should be considered for functional studies in the future.

In the maternal circulation, PAGs have an outstanding long half-life, which varies between different stages of pregnancy and also depends on the test systems applied. In early pregnancy (after induced late embryonic death at days 30–38), the PAG-1 half-life is 3–4 days (Szenci *et al.* 2003). A postpartal half-life of 8.4 days was observed by Sasser & Ruder (1987) and Kiracofe *et al.* (1993). Such a long half-life limits the applicability of PAG-based pregnancy testing if cows are tested within 100 days after parturition for a new pregnancy. Recently, a new ELISA was established (Green *et al.* 2005), which

Figure 5 ((A)–(D)) Western blot of placental tissue of three late pregnant (days 240 and 260) and three term placenta (10 µg/lane). Samples without (–) or with (+) enzymatical release of asparagine-linked glycans were loaded. (A) PAG. Blots were probed with anti-PAG serum (PAG-F4). In late pregnancy, the main PAG isoform has an approximate MW of 66 kDa (big arrowhead) and minor band can be seen at 75 and 56 kDa (small arrowheads). At term, a 64 kDa band (double arrowheads) was the most prominent. The deglycosylated PAG had a MW of 37 kDa (arrows). (B) PRP-I. Main PRP-I bands at late pregnancy and term were at 35 and 34 kDa respectively, and were reduced to 25 kDa after deglycosylation. (C) PHA-L. In late pregnancy, PHA-L binds to PAGs (compare with (A)) with a main band at 66 kDa (big arrowhead; see also Fig. 4A) and minor bands at 75 and 56 kDa (small arrowheads). There is also binding to PRP-I (arrows, see Fig. 4B) and to a 44 kDa glycoprotein, which reacts with the PRP-I antiserum. At term, faint bands (double arrowheads) are seen in the same region (64 kDa) as in (A). (D) DBA. DBA binds to PAGs in the late pregnancy samples (arrowhead marks the 75, 66, and 56 kDa PAG), but not in term placentas. PRP-I is seen as faint band in the late pregnancy samples (arrow).

Figure 6 (A) PHA-L-ELISA with sera of pregnant and non-pregnant cows. The mean OD values are expressed ($\pm$ s.d.). Different superscripts denote statistically different values ($P < 0.05$). (B) DBA-ELISA with sera of pregnant and non-pregnant cows. The mean OD values are expressed ($\pm$ s.d.). Different superscripts denote statistically different values ($P < 0.05$).

overcomes this problem, since it detects PAGs, which have a shorter serum half-life (4.3 days). Protein glycosylation is one important factor, which regulates the half-life of serum proteins. For example, the sulfated GalNAc in lutropin is responsible for its rapid clearance, which is a prerequisite for the pulsatory changes of lutropin concentration (Mi *et al.* 2002). Glycans with terminal GalNAc are potential ligands for the asialoglycoprotein receptor (Coombs *et al.* 2006), which is important for the clearance of asialoglycoproteins from blood. Therefore, the longer postpartal serum half-life of PAGs and perhaps also the dramatic prepartal increase in PAG serum concentration (Sasser & Ruder 1987, Green *et al.* 2005) might be caused by the peripartal loss of terminal GalNAc.

The values of the PHA-L-ELISA at parturition were at the same level as at late pregnancy. This does not correlate to the obvious partial decrease in the placental PAG-expression, which is revealed by the reduced number of partial BNC and the paleness of PAG-bands in western-blot analysis of partial samples. The explanation for this discrepancy could be that the high partial PHA-L-ELISA-values reflect PAGs, which were released into the maternal blood at relatively high rates before parturition, while the histochemical and western analyses show the low quantities, which remain in the placenta. One astonishing finding is that, in contrast to other test systems, no peripartal peak of PAG-concentration is seen in the

PHA-L-ELISA. Possibly, the PHA-L-ELISA recognizes only a subpopulation of PAGs, which has a serum profile different from PAGs, detected by other test systems. Such differences could be advantageous for an application in pregnancy testing.

Our data demonstrate a substantial peripartal switch of glycosylation of secretory glycoproteins that are produced by the BNC in the bovine placenta. On these proteins, terminal GalNAc on asparagine-linked glycans disappears prior to parturition. A similar trend with reduced partial quantities of DBA-binding PAGs in maternal serum was revealed by the lectin-ELISA. The functional background of this change is still obscure, but a modulation of receptor binding or regulation of serum half-life appears possible.

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