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Pregnancy-associated glycoproteins in cows with retained fetal membranes

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ABSTRACT

In cows, retained fetal membranes (RFM) are a major problem in reproduction. The timely detachment of fetal membranes after parturition requires well coordinated maturation processes in the placenta. One feature of placental maturation in cows is a prepartal decline in the number of binucleate trophoblast giant cells (BNC) in the fetal chorion. Pregnancy associated glycoproteins (PAGs) are a group of proteins, produced by trophoblast cells in artiodactyls. We studied aspects of PAG expression in cows with and without RFM. The numerical density of PAG-positive immunostained BNC in placentomal samples, collected from cows with normal expulsion of fetal membranes (n = 20) and cows with RFM (n = 20) was determined. The number of PAG-positive BNCs was significantly higher in cows with RFM, compared to controls. The concentration of PAGs in maternal serum in prepartum, intrapartum, and postpartum cows was measured (RFM n = 20; controls n = 68). No significant differences between RFM and controls were detected. Microarray analysis of placental PAG mRNA expression was done with two types of microarrays: Affymetrix (RFM n = 20; controls n = 20) and Agilent (RFM n = 8; controls n = 8). Both microarrays showed a significantly higher expression of modern PAGs in RFM cases.

Our results show that the expression of modern PAGs, which are produced by BNCs and are secreted into the maternal organism, are differentially expressed in RFM. Although the concentration in peripheral maternal blood did not differ between RFM and controls, the local concentration in the placenta is likely to be higher in RFM cases. This suggests the possibility of local regulatory roles of PAG in the release of bovine fetal membranes.

1. Introduction

Retained fetal membranes (RFM) in cattle is defined as the failure to release the fetal membranes within 12 or 24 h after expulsion of the calf [1]. It severely affects the subsequent reproductive capability in dairy cows and consequently leads to significant economic losses [2]. The incidence of RFM after normal pregnancy is about 4% [3], but the frequency of RFM can be severely increased by several risk factors [4–7]. The prerequisite for a timely detachment of the fetal membranes are maturation processes inside the placentomes. At the feto-maternal interface the main morphological characteristics of this maturation are the attenuation of the maternal caruncular epithelium and the decline in the number of fetal binucleate trophoblast giant cells (BNCs) [8]. The BNCs are large migratory cells with a complex developmental history. These cells are continuously formed in the trophoblast epithelium [9]. They become binucleate by acytokinetic mitoses [9] and undergo polyploidisation [10] and maturation, during which they accumulate large quantities of cytoplasmic granules. After the fusion with a uterine epithelial cell, the contents of these granules are released into the maternal endometrial connective tissue [11]. During most time of pregnancy, the frequency of BNC in cattle is about 20% of all trophoblast cells [12]. The frequency of BNC declines towards parturition, but in cows with RFM this decline is

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significantly reduced [8].

Pregnancy associated glycoproteins (PAGs) are one major group of the secretory proteins produced by ruminant trophoblast cells [13]. The PAGs form a large polymorphic family of glycoproteins with more than 20 members expressed in the bovine trophoblast. Based on phylogenetic analysis of the sequences, PAGs can be subdivided into two groups: the 'ancient' and the 'modern' PAGs [14]. The PAGs show a complex expression pattern, which changes during pregnancy [13]. Expression of 'modern' PAGs is generally limited to BNCs, while the ancient PAGs are predominantly expressed in uninucleate trophoblast cells (UNCs) [13,15]. Using antibodies, which were designed to differentiate individual PAGs in immunohistochemistry, a recent study demonstrated that the expression pattern of PAGs is more complex than previously thought [15]. Ancient PAG11 was localized in intercotyledonary BNC and in BNC of the arcade region of the placentomes, whereas ancient PAG2 was predominantly localized in cotyledonary UNCs [15].

Members of the group of modern PAGs can be employed as biochemical pregnancy markers in the cow's blood or milk [16]. Despite the prepartal decline of BNC number, the concentration of PAGs in maternal plasma peaks around parturition. Tefera et al. [17] showed that in cows with RFM the PAG concentrations in maternal serum during the first 32 days post partum are higher than in controls.

In the present study we analysed the expression of PAGs in placentomal samples from cows with and without RFM by immunohistochemistry and microarray analysis. The PAG concentrations in serum samples were measured by a radio immuno assay. The aim of the study was to elucidate aspects of PAG expression in relation to RFM.

2. Material and methods

2.1. Selection of cows and tissues collection

The present study used materials from a larger project. The animal procedures were approved by the Landesamt für Verbraucherschutz, Landwirtschaft und Flurneuordnung, Frankfurt (Oder), Germany, file number 23-2347-A-25-1-2009.

The studies were performed at a commercial dairy farm in Germany. During the time of sampling 900–1100 cows were in milk, with an average yearly milk yield of 10,489 kg.

The placental tissue was sampled as described previously [18]. In brief, three placentomes were extracted with an effeminator after Reisinger, modified after Richter [19], via vaginalis within 15 min after rupture of the umbilical cord. Every placentome was cut perpendicular to the luminal surface in 0.5 cm thick slices, which were placed with the right cut surface facing downwards on a sterile RNase-free surface. The slices were covered with a grid (1 × 1 cm) and every point hitting the visual feto-maternal connection was counted. These connecting points were divided by three resulting in n = x, and a random number between 1 and x was determined, where tissue samples were collected. Tissue cuboids (0.5 × 0.5 × 0.75 cm) were cut out and divided into three parts of approximately 0.5 × 0.5 × 0.25 cm. The middle part was transferred into RNAlater (Ambion, Huntingdon, Cambridgeshire, UK), incubated overnight at 4 °C, and then stored at -20 °C until further processing. The other samples were fixed for 24-48 h in formalin (3.7%) and paraformaldehyde (4.2%), respectively, and routinely processed and embedded in paraffin.

Blood samples were taken from cows at three different stages: prepartum (68 ± 27.19 h before expulsion of the calf), intrapartum (0.13 ± 0.68 h after expulsion of the calf), and postpartum (46 ± 11.06 h after expulsion of the calf). Blood samples were taken from the coccygeal vein, using vacutainer tubes (with clot activator, Becton Dickinson, Heidelberg, Germany). The blood was stored for up to 2 h at room temperature, centrifuged (4,472 g; 5 min) and serum aliquots were stored at -20 °C.

2.2. Immunohistochemistry (IHC)

2.2.1. Staining procedures

The immunohistochemical analysis was carried out on three paraffin sections (5 μm) per animal, one from each sampled placentome. Sections were dewaxed in xylol, rehydrated in descending concentration of ethanol and rinsed in distilled water. Antigen retrieval was performed by heating sections in 0.01 M citrate buffer (pH 6.0) 3 times for 5 min in a microwave oven at 600 W. Sections were blocked with 10% goat serum for 20 min. Subsequently the slides were incubated with a polyclonal rabbit anti-PAG serum (R♯497, raised against bovine 67 kDa PAG) [20] diluted 1:4000 in PBS, containing 1% BSA, in Shandon's coverplates overnight. Slides were washed two times for 5 min in PBS and incubated for 30 min with biotinylated Goat Anti-Rabbit IgG Antibody (1:100, Vector Laboratories) in PBS. After 5 min washing in PBS, the slides were incubated with streptavidin/biotin-peroxidase complex (Vector Laboratories). The slides were then washed in PBS, stained with DAB (Diamminobenzidine, Sigma)/ammonium nickel (III) sulfate-solution, counterstained with hematoxylin and coverslipped. In negative controls non-immune rabbit serum (Dako, Hamburg, Germany), diluted 1:4000 in PBS was used.

2.2.2. Quantitative analysis

The stained sections were analysed with a LEICA light microscope at a final magnification of ×200. During image acquisition and counting the investigator (R.H.) was not aware which sections were from controls and which were from RFM-cases. Digital images were taken from the sections at locations of 1 mm distance, both in the x- and y-direction in a random systematic way. This was achieved by moving the stage control of the microscope in 1 mm steps in the x- and y direction between image acquisitions. The quantification of PAG-positive BNC in the digital images was done with a free software (STEPanizer [21]). An unbiased counting frame, which included 9 test points, was used (Fig. 1). In each field the proportion of fetal and maternal tissues were quantified by a point counting method. The number of round PAG-positive cells, which had at least one nucleus in the section plane and were located in fetal tissue, was counted as BNCs. For each animal the density of BNC in fetal tissue was calculated (BNCs/mm^2 of fetal tissue area). Results are given as mean ± SD. The achieved mean values for each experiment were compared to the control (Student's t-test, considering values of $p < 0.05$ significant).

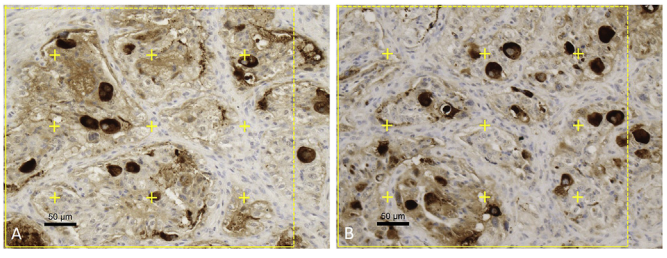

Fig. 1. PAG immunohistochemistry of bovine placentomes at parturition of controls (A) and of cows with RFM (B). The PAG-positive BNCs in fetal tissue were quantified, using an unbiased counting frame (yellow lines). The 9 test points (yellow crosses) were used to determine the fraction of fetal tissue. Scale bars = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)