Placenta 57 (2017) 216–222

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Glycosylation and immunocytochemistry of binucleate cells in pronghorn (*Antilocapra americana*, Antilocapridae) show features of both Giraffidae and Bovidae

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ARTICLE INFO

Article history:
Received 23 June 2017
Received in revised form
14 July 2017
Accepted 17 July 2017

Keywords:
Pronghorn
Lectins
Placenta
Binucleate cells
Giraffidae
Bovidae

ABSTRACT

Although the pronghorn (*Antilocapra americana*) resembles an antelope, its nearest relatives are the giraffe and okapi. In this study we have examined the placentae of 6 pronghorns using lectin- and immunocytochemistry to identify giraffid and bovid features. Binucleate cells (BNC) of the placenta exhibited features intermediate between those of the giraffe and bovine; *Dolichos biflorus* agglutinin binding – strong in the bovine BNC and absent in the giraffe – was evident in only a subpopulation of BNC while binding to blood vessels, as in the giraffe. Binding of *Phytolacca americana* agglutinin resembled that of the giraffe and okapi whereas many other glycans were found in all four clades. PAG antigens were similar to bovine and okapi but not giraffe. In summary, although the pronghorn outwardly resembles an antelope, placental BNC show giraffid features. Although each clade has its own individual characteristics, there are far more similarities than differences between them, emphasizing the common ancestry of all four clades.

1. Introduction

The pronghorn (*Antilocapra americana*, Antilocapridae) is an artiodactyl mammal indigenous to North America and is often referred to locally as an antelope. However, phylogenetically Antilocapridae occurs in a superfamily, Giraffoidea, with giraffes and okapi (Giraffidae). In the Pleistocene period, there were 12 taxa of the family Antilocapridae but now the pronghorn is the only extant species. It bears characteristic forked horns [1] that are covered in skin as in giraffes, but in the pronghorn this becomes a keratinous sheath which is shed and regrown on an annual basis [2].

An important feature of the ruminant placenta is the fetal chorionic binucleate cell (BNC) which migrates across the microvillous membrane to fuse with maternal cells, forming fetomaternal trinucleate cells or syncytial masses [3]. These binucleate cells contain heavily glycosylated granules which have been shown to contain placental lactogens which, on migration, pass over to the maternal circulation [4]. Recently, we showed that the placental binucleate cell (BNC) of the giraffe and okapi has a different pattern of glycosylation from other ruminant BNCs that we have studied [5]: greater malayan chevrotain (Tragulidae); fallow deer, red deer, Chinese water deer (Cervidae), domestic goat, springbok, impala, domestic cow and sheep (Bovidae) with little or no expression of terminal αN-acetylgalactosamine bound by *Dolichos biflorus* and *Vicia villosa* agglutinins which instead bind to placental blood vessels [6]. We also demonstrated different patterns of protein expression in the BNC [7].

It appeared that Giraffidae BNC developed different pathways in their glycan biosynthesis and protein expression following their split from the Bovidae, with further differences evolving as okapi and giraffe diverged from each other. Because the pronghorn-

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http://dx.doi.org/10.1016/j.placenta.2017.07.011
0143-4004/

giraffe clade (or Antilocapridae-Giraffidae clade) diverged from Bovidae [8], it is possible that pronghorn BNC might be different from bovine BNC. We therefore examined placentae from six specimens in order to characterise the glycosylation and protein expression of the binucleate cells and to compare them with those from the giraffe, okapi and bovine.

2. Materials and methods

2.1. Animals

All procedures for collection of animals and tissues were approved by the Fort Keogh Institutional Animal Care and Use Committee (IACUC No. 032415-1). The six pronghorn placental samples (Table 1) were collected and fixed within 20 min after death as part of a wider investigation of pronghorn biology carried out in eastern Montana. Whole placentomes, consisting of fetal cotyledons in close association via microvillous interdigitation with maternal caruncles that form button-like outgrowths on the surface of the uterus, were placed at 4 °C in Surgipath I B F fixative (isopropyl alcohol, methanol, barium chloride and <3% formaldehyde, Leica Biosystems Inc, Buffalo Grove, IL 60089, USA) for 14 days. Central slices of each pronghorn placentome were then cut, put into fresh Surgipath fixative and sent to the UK. The six pronghorn samples had crown rump lengths (from the top of the head to the rump just above the tail) of one of twin fetuses extending from 277 to 318 cm (approximately 160–190 days old, gestation being 240 days). “Matchstick” samples from the central region of each placentome slice from maternal to fetal edge were embedded in epoxy resin.

2.2. Lectin histochemistry

Sections 0.75 μm thick were cut, deresinated in sodium ethoxide, and stained with a panel of 23 biotinylated lectins (see Table 2 for details) at 10 μg/ml as previously described [9] except that SNA-1 was used at a concentration of 50 μg/ml. Some deresinated sections were treated with Type VI neuraminidase (Sigma, from *Clostridium perfringens*) in 0.2 M acetate buffer, pH 5.5, with 1% (w/v) calcium chloride at 37 °C for 2 h [10] to remove terminal sialic acid prior to lectin staining. This revealed glycan residues that were previously masked by the sialic acid. Controls were as previously described [9]. For BSA-II staining for amylase-sensitive glycogen [11], the trypsinisation step was omitted and staining was controlled by digestion in 1% amylase (Sigma, UK) for 20 min at 37 °C following by washing in running water for 10 min. Sections were compared with those of giraffe, okapi and bovine (*Bos taurus*) from our previous study [6] which had been embedded and stained with the same panel of lectins in a similar manner. Subsequently, a series of 8 serial sections was cut from each pronghorn specimen and stained with lectins of particular interest (UEA-1, l-PHA, DBA, VVA, ECA, PAA and WGA) so that specific BNC cells could be tracked to determine glycan characteristics for individual cells. Specific BNC and villi were visualized for the same tissue by overlaying identical fields following staining procedures.

Table 1
Pronghorn twin fetal characteristics.

Specimen number	Crown-Rump Lengths (mm)	Average weight (g)
1	307/301	1134
2	290/290	1025
3	288/290	1080
4	284/290	995
5	283/277	900
6	318/295	1000

2.3. Immunocytochemistry

Samples were postfixed overnight in 4% (para)formaldehyde in PBS with or without 1% glutaraldehyde before epoxy embedding. Semithin sections were cut, picked up on cover glass squares treated with APES and deresinated in sodium ethoxide. The cover glass squares were then floated section side down on drops of antibody followed by immunogold colloid (goat anti-rabbit G5, Jackson Immunoresearch Labs, USA) then intensified with silver reagent (Aurion, Wagenigen, Netherlands). The antibodies used were to Pregnancy Associated Glycoproteins (PAGs) Ovine PAG-1 and Bovine PAG-2 [12] used at a dilution of 1:1000, SBU-3 [13,14], bovine Placental Lactogen (bPL) and prolactin (PRL) [7], all at 1:100. Controls with buffer substituted for antibody showed no significant labelling. The two postfixations produced similar results.

3. Results

3.1. General structure of the placentome

As in all ruminant placentomes, development starts from a flat apposition of trophoblast and uterine epithelium. Mutual growth of the fetal and maternal layers produces placentomes consisting of chorionic villi interdigitating with maternal villi. The fetal digitiform primary villi branch off short, leaf-like secondary villi at right angles to the primary ones while the endometrial villi contain secondary villi to house the secondary fetal villi (Fig. 1A and B). The maternal villi are covered with uterine epithelium underneath which are small blood vessels running along the stroma, while the trophoblast covers primary fetal villi that have a similar subepithelial capillary network with a broader and more loosely arranged mesenchymal core through which run large blood vessels.

The bases of the fetal villi have more regularly arranged columnar cells than cells on the villus sides, similar to those of the arcade regions at the very tips of the villi which are continuous with the flat intercotyledonary area of the placenta (Fig. 1A) on the fetal side, which joins up adjacent cotyledons.

3.2. Lectin histochemistry of binucleate cells (BNC)

Lectin histochemistry of BNC is summarised in Table 2. There was little detectable difference in the results between specimens and two (specimens 1 and 6) are presented in the table as typical examples. There was, however, great variability both in the distribution of BNC stained (Fig. 1C and D) and in their staining intensity with the various lectins (Fig. 1E–H). BNC in the basal regions tended to be smaller than those in the secondary villi themselves while in the arcade region they were large with rather dispersed granules. LEA (Fig. 1C), ECA with neuraminidase, WGA, and PAA both with or without neuraminidase treatment stained the majority of BNC throughout the placentome. In contrast to this, DBA (Fig. 1D) and VVA, did not stain the BNC in in the basal areas or arcade region though staining of cells in secondary villi was strong. This was also evident to some extent with l-PHA, ECA without neuraminidase (Fig. 1E, H) and WFA, where some generally small stained cells were evident in the basal regions but there were more at the tips of the secondary villi. Conversely, the few cells that bound UEA-1 and MAA were mainly found in the basal areas and arcade region rather than in the secondary villi and examination of serial sections revealed that the cells that bound UEA-1 more strongly were invariably those with no DBA or VVA staining; likewise strongly stained DBA/VVA cells were generally negative with UEA-1 (Fig. 1F and G). Comparison of lectin binding of the same six cells with l-