Investigations on Pregnancy-Associated Glycoproteins in the Cow

Glycoproteins expressed in the ruminant placenta have been isolated and characterized during the last two decades: PSPB (Butler *et al*., 1982); PAG (bPAG1, PAG I_{67}, bovPAG 1) (the number in the subscript refers to molecular weight in kDa) (Zoli *et al*., 1991); PAG_{55}, PAG_{59}, PAG_{62} (Garbayo *et al*., 1998).

Pregnancy specific proteins were identified and characterized as early as 1982 by Butler *et al*. (1982) as PSPA and PSPB. The first, PSPA was considered as corresponding to bovine α-fetoprotein and thus was not strictly specific for pregnancy. The second one, the PSPB later named bPSPB was rapidly proposed as a good marker, detectable in peripheral blood, for pregnancy diagnosis and follow up (Sasser *et al*., 1986). In 1991 Zoli *et al*. (1991) isolated and characterized placental glycoproteins having a molecular weight of 67 kDa and four isoelectric points (4.4, 4.6, 5.2 and 5.4) with the following NH_2-terminal sequence: Arg-Gly-Ser-x-Leu-Thr-Thr-His-Pro-Leu. Soon after, molecular cloning investigations on bovine and ovine placenta, showed that PAGs belonged to the aspartic proteinase family sharing sequence identity with pepsin (57%), pepsinogen (49.5%), cathepsin D (58%) and E, chymosin (42.5%) and renin (Xie *et al*., 1991b; Xie *et al*., 1994; Xie *et al*., 1995). Distance phylograms showed that the PAG gene family probably diverged from the enzymatically functional aspartic proteinases (Xie *et al*., 1997). Guruprasad *et al*. (1996) suggested that the bilobed three-dimensional structure of bovPAG-1 was similar to that of pepsin and chymosin, moreover bovPAG-1 was able to bind pepstatin to its binding site like the other enzymatically active aspartic proteinases. In 1992, Lynch *et al*. found a high cDNA sequence identity between PSPB and bovPAG I_{67} and classified the PSPB in the same aspartic proteinase family. Only the PAG I_{67} was characterized in term of carbohydrate and sialic acid content (Zoli *et al*., 1991). In the following years other pregnancy-associated glycoprotein sequences were identified in the ruminant placenta by molecular biology studies (Xie *et al*., 1997; Green *et al*., 2000).

In 1998, some PAG molecules purified from caprine placental extracts were characterized as PAG_{55} (NH_2-terminal sequence: Ile-Ser-Ser-Pro-Val-Ser-x-Leu-Thr-Ile), PAG_{59} (NH_2-terminal sequence: Arg-Gly-Ser-x-Leu-Thr-Thr-Leu-Pro-Leu), PAG_{62} (NH_2-terminal sequence: Arg-Asp-Ser-x-Val-Thr-Ile-Val-Pro-Leu) (Garbayo *et al*., 1998).

Molecular biology investigations showed that there are probably more than 100 PAG genes in the ruminant genome (Xie *et al*., 1997) most of them being expressed in the superficial layers of the placenta. For example, in the bovine species, two different patterns of expression were found: some of the PAGs like bovPAG-2, -8, -10 and -11 were expressed in the mono- and binucleate cells of the trophectoderm, while other proteins like bovPAG-1, -4-7, -9 were localized in the binucleate cells (Green *et al*., 2000). Molecular biology investigations also concluded, that during certain stages of pregnancy some PAGs were expressed, while others were absent (Garbayo *et al*., 1999; Green *et al*., 2000). In trophectoderm of conceptuses removed on Day 25, 45, 60, 88, 150, 250 after fertilization, Green *et al*. (2000) detected the mRNA encoding for PAG I_{67} from Day 45, while the mRNA for bovPAG-4 and bovPAG-9 were predominantly present at Day 25. An important question remained unanswered: were PAGs secreted into the maternal circulation earlier and is it possible to evidence their presence in the maternal blood. Surprisingly, compared to molecular biology studies, the biochemical approaches allowed the purification of a few different molecules.

Three different radioimmunoassay systems (RIA 1, RIA 2 and RIA 3) using antisera produced against PAG I_{67} (RIA 1), PAG_{55+62} (RIA 2) and PAG_{55+59} (RIA 3) were used in this work. Two of these additional RIA systems (RIA 2 and RIA 3) were developed and validated in this study for the detection of pregnancy-associated glycoproteins. Their sensitivity (in term of minimal detection limit), accuracy, precision were characterized and compared to those of RIA 1. The specificity of the three RIA systems against the commercially available, enzymatically active members of the aspartic proteinase family like pepsinogen, pepsin, chymosin, rennet, cathepsin D, renin were determined (Perényi *et al*., 2002b). These products were tested in a wide concentration range (10 ng/ml to 1 mg/ml). Pepsinogen crossreacted in RIA 1, RIA 2 and RIA 3 over 1 mg/ml, 50 µg/ml and 500 µg/ml concentrations, respectively. In the presence of pepsin, crossreaction was observed in RIA 1, RIA 2 and RIA 3 over 1 mg/ml, 500 µg/ml and 1 mg/ml concentrations, respectively. Chymosin caused crossreaction in RIA 2 and RIA 3, rennet crossreacted in RIA 2. Renin and cathepsin D did not decrease the binding of the tracer to antisera more, than that of the minimal detection limit. As the plasma / serum concentrations of the examined aspartic proteinases reported in the literature were outside the concentration range where crossreaction was observed, it can be concluded that these RIA systems were specific for the detection of PAGs in biological fluids.

The three RIA systems were used in order to measure the PAG concentration in plasma samples withdrawn from pregnant cows and heifers throughout different periods following artificial insemination (AI) (Perényi *et al*, 2002a). These systems were able to detect PAG molecules in the maternal blood as early as 21 days after AI in different concentrations (RIA 1: 0.43 ± 0.24 ng/ml, mean±SD; RIA 2: 0.48 ± 0.24 ng/ml; RIA 3: 0.64 ± 0.37 ng/ml). On Day 32 and 42 RIA 2, (4.30 ± 1.32 ng/ml, 5.56 ± 1.95 ng/ml) and RIA 3 (4.17 ± 1.15 ng/ml, 5.60 ± 1.89 ng/ml) presented significantly ($p<0.0001$) higher PAG concentrations than those of RIA 1 (2.43 ± 0.81 ng/ml, 4.01 ± 1.48 ng/ml), respectively. After Day 21, significant correlations ($p<0.0001$; $r\geq0.929$) were determined between the three systems. This study clearly indicated that the ability of a RIA test to recognize PAG molecules in the maternal blood can be improved by carefully selecting the antiserum. As the PAG I_{67} transcripts became first detectable 45 days after AI, our observations, suggest that all the three RIA systems are probably detecting PAGs different and earlier expressed than PAG I_{67}.

We also aimed to determine PAG concentrations in samples removed frequently from pregnant heifers. Using three PAG RIA systems, we presented two partial and five complete individual PAG profiles from our experimental animals. Five of the profiles were representing normal ongoing pregnancies, whereas two of them pregnancies with abortion and embryonic loss. The ratios between the concentrations determined by the newly developed RIA systems and RIA 1 system were calculated. Decrease could be observed during the first 60 days of pregnancy in these ratios. The analysis of individual PAG profiles presented showed that PAG molecules secreted in the maternal blood between 21 and 60 days after AI were better recognized by RIA 2 and 3 systems.

From our observations it can be suggested, that further investigations should be carried out in order to isolate and characterize the PAG molecules released in early pregnancy and detected with higher efficiency with RIA 2 and RIA 3. RIA 2 and 3 systems would be efficient tools to follow the immunoreactive PAG containing fractions during a purification procedure.

The individual PAG profiles suggesting that such a purification should be performed using cotyledons collected from cows during the first 60 days of their pregnancy.

In a collaborative study 268 embryo recipients were followed by clinical observation and blood sampling at short time interval (every three day from Day 7 until Day 35, then once a week until Day 119). In this study PAG and P4 profiles are presented which were determined from maternal blood. The analysis of the profiles allowed to classify the recipients into 3 groups: Group A: abnormality first occurred in the P4 profile; Group B: abnormality first occurred in the PAG profile; Group C: abnormality occurred at the same time in the P4 and PAG profiles. Pregnancy failures classified as type A were probably originating from the mother, while type B pregnancy failures from the embryo. In the case of type C pregnancy failures the reason of the embryonic loss was in the mother or/and in the embryo. By the retrospective analysis of these profiles valuable informations were gathered concerning to the origin and the pathogeny of the pregnancy failure.

The determination of P4 and different PAG molecules in plasma samples collected frequently combined with ultrasonographic detection of the embryonic heart beating and rectal palpation could be very efficient tool in the pregnancy follow-up.

The analysis of P4 and PAGs profiles from maternal blood of animals receiving embryos produced in vitro using different culture media allows to investigate the influence of those culture media on the development of the embryo and the trophoblast.

In conclusion, new efficient tools for the pregnancy detection and follow up in cattle were developed in the first part of our study. The efficiency of these RIA systems was verified in normal and failed pregnancies followed by frequent blood sampling in our experimental farm. These assays were used to characterize a large series of pregnancies after transfer of embryos of different origin.