Abstract Preimplantation embryos of several species are surrounded by an extraembryonic matrix (often simply named zona pellucida) until briefly before implantation. All signals of the early embryo-maternal dialogue have to pass this matrix and therefore are detectable inside. We investigated the protein pattern of the extraembryonic matrices of 3–6-day-old rabbit embryos by two-dimensional gel-electrophoresis. Using 35S-methionine incorporation, embryonic proteins were labelled and could be distinguished from maternal proteins. Furthermore, the presence of three different proteins (insulin-like growth factor-binding protein-3, uteroglobin, haptoglobin) within the matrices of day-6 embryos was investigated by Western blot analysis. The pattern and numbers of protein spots detected was clearly dependent on the time of embryonic development. Of all proteins detected, 19.3% and 33% are of embryonic origin (day 5 and day 6, respectively). At day 4 the zona proteins are no longer detectable, reflecting the degradation of the zona pellucida. From day 4 to day 5 proteins detectable within the extraembryonic matrices increase enormously. This demonstrates that embryo-maternal signalling accelerates at least 2 days before implantation. Insulin-like growth factor-binding protein-3, uteroglobin and haptoglobin are part of the early signalling as shown by Western blot analysis. Insulin-like growth factor-binding protein-3 could be detected as one spot at 38 kDa pI 6.1, uteroglobin at 8 kDa pI 6.0 and haptoglobin as two spots/isoforms at 36/38 kDa pI 5.8 and pI 6.0. These results demonstrate that extraembryonic matrices serving as a mailbox are a valuable tool for investigating early embryo-maternal signalling.

Keywords Embryonic coats · 2D SDS PAGE · Embryo-maternal signalling · Zona pellucida

Abbreviations 2D SDS PAGE: two-dimensional polyacrylamide gel electrophoresis · IEF: isoelectrical focusing · IGBP3: insulin-like growth factor-binding protein-3 · pI: point of isoelectricity

Introduction

All known preimplantation embryos are surrounded by an extracellular matrix. Furthermore, fossil embryos, more than 570 million-years-old, have been shown to be covered by an extraembryonic matrix (Xiao et al. 1998). While ruminant and pig embryos hatch from their coats after about 50% of their preimplantation lifespan (53%/cattle, 50%/pig), horse, cat, dog, mice, human and rabbit embryos lose them only briefly before attachment to the endometrium (Betteridge 1995).

The extraembryonic matrix of the rabbit is transformed continuously during early embryonic development. Oocytes as well as zygotes are surrounded by the zona pellucida. Thereafter, oviductal, uterine and embryonic secretions are incorporated/deposited into/onto the zona pellucida, which is represented in the rabbit by the formation of different layers of extraembryonic matrices (Denker 2000; Herrler and Beier 2000). Oviductal secretions covering the zona pellucida are called the mucoprotein layer. Uterine secretions are represented by the gloilemma. Around day 4 the zona pellucida is replaced by the neozona composed of embryonic and uterine components (Böving 1963; Denker and Gerdes 1979; Leiser and Denker 1988; Fischer et al. 1991). These layers can be distinguished by electron as well as light microscopy. The first reorganisation of the extraembryonic matrix can be observed some hours after entry into the oviduct (deposition of the mucoprotein layer), the second at day 4 (replacement of the zona pellucida by the neozona) and finally at day 6 p.c. by deposition of the gloilemma, thus demonstrating the dynamics of the consecutive
changes of the matrix. Oviductal secretions are not only layered onto the zona pellucida, they also penetrate this layer and are found within the perivitelline space (perivitelline matrix; Fléchon 1974; Talbot and DiClarantonio 1984; Dandekar and Talbot 1992; Dandekar et al. 1995), providing evidence that maternal secretions penetrate the coats to reach the embryo. Therefore, maternal/embryonic proteins, which are directed towards the embryo/mother, should be detectable within the embryonic coats. As the extraembryonic matrix surrounds the embryo until briefly before adhesion to the endometrium, all messages involved in embryo–maternal signalling have to pass this special embryo–maternal interface and therefore should be detectable within this matrix, thus serving as mailbox of the embryo–maternal dialogue.

In this investigation we determined the protein pattern of preimplantation rabbit extraembryonic matrices with the aim of obtaining new insights into early embryo–maternal signalling. Day 3–6 embryonic coats were separated from the conceptus by microsurgery and proteins were dissociated by two dimensional polyacrylamide gel electrophoresis. On inspection of the protein pattern, the question as to the origin of these messages arose. By 35S-methionine incorporation, all embryonic proteins were labelled and could be distinguished from those of maternal origin. Western blot analysis identified three different maternal proteins [insulin-like growth factor-binding protein-3 (IGFBP3), uteroglobin, haptoglobin] within the extraembryonic matrices. The identity of some spots was discussed with respect to their estimated molecular weight and point of isoelectricity.

**Experimental procedures**

Animals and sample collection

All experiments on animals were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals and were run with the permission of the District Government at Cologne, Germany (AC 39 15/95). Embryos were recovered from New Zealand White female rabbits ranging in age from 6 months to 8 months. They were housed individually in air-conditioned rooms (25 °C, 45% relative humidity) under a 12L:12D light cycle (0700–1900 hours) and were fed with a commercial diet and water ad libitum.

To induce ovulation, 75 IU hCG (Primogonyl, Schering, Berlin, Germany) were administered i.v. directly after mating. To induce multiple follicular growth, the rabbits received a single dose of 100 IU PMSG s.c. (Intergonan, Intervet, Tönisvorst, Germany). Embryos, 20 of day-4, 10 of day-5 and 5 of day-6 embryos were cultured for 4 h in a mixture of 1:1 DMEM/RPMI, without glutamine and methionine (ICN, Eschwege, Germany), supplemented with 3 mmol glutamine, 30 µmol methionine and 5.5 MBq 35S-methionine per ml (0.13 µmol; 43.5 TBq/mmol; ICN, Eschwege, Germany). We have chosen this gradient as by the 10% gel, small proteins down to 6 kDa (area of most growth factors) get separated very well, and by the 18% gel, the same is true for the bigger proteins up to 100 kDa (area of binding proteins and carrier). Stripes were equilibrated in DTT (100 mg/10 ml buffer, 15 min) and iodoacetamide (480 mg/10 ml buffer, 15 min; buffer: 60 mM NH4HCO3, 0.1% SDS, 0.8 mM EDTA, 0.15 mM bromphenolblue, 0.05 M Tris/Cl pH 6.8). Thereafter, stripes were placed onto the 5% stacking gel. In parallel, 2 µl of a protein standard (marker 4+5, Serva Heidelberg, Germany) was separated. To determine the pl, 10 µl of a 2D standard (BioRad München, Germany) was focused for each IEF stripe charge. This gel and the kDa marker on each gel were used to determine the kDa and pl scales for each 2D gel. Running conditions for the second dimension were as follows: 60 min 200 V 25 mA 30 W, 4.5 h 600 V 30 mA 30 W at 15 °C. Per embryonic developmental stage, 3–5 gels were run.

For determination of the protein patterns on days 3–6, gels were silver stained following the protocol of Blum et al. (1987). Afterwards, gels were equilibrated for 20 min in glycerol/ethanol (10% v/v, 30% v/v). For drying, gels were fixed in between two cellophane foils in a frame.

To edit and compare the gels, they were digitalized by scanning (Immage Station 440, Kodak, Stuttgart, Germany). Those files where further investigated by using Melanie3 (Genebio, Gen-eva, Switzerland).

Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D SDS PAGE) was performed as described by Görg et al. (1988). Stripes for isoelectrofocusing (IEF) with a point of isoelectricity (pl) of 4–10 were prepared according to the protocol of Görg et al. (1988; using Immobelines pK 3.6–9.3, Amersham Pharmacia, Freiburg, Germany; thickness 0.5 mm), dried and stored at –20 °C. Samples were dissolved in 30 µl sample buffer (9 M urea, 32.5 mM chaps, 9 mM PMSE, 2% v/v mercaptoethanol, 2% v/v ampholine; O’Farrell 1975). Focussing was performed using the Multiphor II-system (Amersham Pharmacia, Freiburg, Germany) at 20 °C. Focussing conditions: 30 min 150 V 1 mA 1 W, 30 min 300 V 1 mA 1 W, in total about 18,000 V hours. Focused stripes were stored at –70 °C prior to separation in the second dimension.

For the second dimension, 0.5 mm 10–18% resolving gels were prepared using gelbond foils (Amersham Pharmacia, Freiburg, Germany). We have chosen this gradient as by the 10% gel, small proteins down to 6 kDa (area of most growth factors) get separated very well, and by the 18% gel, the same is true for the bigger proteins up to 100 kDa (area of binding proteins and carrier). Stripes were equilibrated in DTT (100 mg/10 ml buffer, 15 min) and iodoacetamide (480 mg/10 ml buffer, 15 min; buffer: 60 mM NH4HCO3, 0.1% SDS, 0.8 mM EDTA, 0.15 mM bromphenolblue, 0.05 M Tris/Cl pH 6.8). Thereafter, stripes were placed onto the 5% stacking gel. In parallel, 2 µl of a protein standard (marker 4+5, Serva Heidelberg, Germany) was separated. To determine the pl, 10 µl of a 2D standard (BioRad München, Germany) was focused for each IEF stripe charge. This gel and the kDa marker on each gel were used to determine the kDa and pl scales for each 2D gel. Running conditions for the second dimension were as follows: 60 min 200 V 25 mA 30 W, 4.5 h 600 V 30 mA 30 W at 15 °C. Per embryonic developmental stage, 3–5 gels were run.

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**35S-methionine incorporation**

Day-4, day-5 and day-6 embryos were cultured for 4 h in a mixture of 1:1 DMEM/RPMI, without glutamine and methionine (ICN, Eschwege, Germany), supplemented with 3 mmol glutamine, 30 µmol methionine and 5.5 MBq 35S-methionine per ml (0.13 µmol; 43.5 TBq/mmol; ICN, Eschwege, Germany). We choose a culture time of 4 h as Nieder and Marcon (1987) have shown that during this time of culture, the protein synthesis is linear and a damage of cells by radiolysis is unlikely. Culturing day 12 horse embryos under the same conditions, we were able to show that such embryos grow very well (Herrler et al. 2000). Following culture, embryonic coats were harvested as described above and stored at –20 °C. Samples were separated by 2D SDS PAGE not more than 3 weeks later and dried gels were exposed to X-ray films at –70 °C (X-Omat AR; Kodak, Stuttgart, Germany). In parallel, a second, non-radioactive sample was separated under the same conditions by 2D SDS PAGE not more than 3 weeks later and dried gels were exposed to X-ray films at –70 °C (X-Omat AR; Kodak, Stuttgart, Germany). In parallel, a second, non-radioactive sample was separated under the same conditions by 2D SDS PAGE and silver stained. Both gels were compared to determine the appropriate 35S-labelled spots on the silver-stained gel.

Western blot analysis

For Western blot analysis 2D gels were run as described before. Gels were cast onto the hydrophobic side of the gelbond foil...