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Classics Revisited

Localisation of epidermal growth factor (EGF), its specific receptor (EGF-R) and aromatase at the materno-fetal interface during placentation in the pregnant mare

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ABSTRACT

Introduction: Implantation and placentation in the mare does not commence until as late as day 40 after ovulation. The reasons for this and the growth factors and/or hormones which drive placentation when it does finally occur are of considerable academic and practical interest.
Method: Placental interface tissues recovered from 11 accurately aged and perfused-fixed horse uteri between 20 and 68 days of gestation were stained immunocytochemically for Epidermal Growth Factor (EGF), its specific receptor (EGF-R) and for the steroid hormone enzyme, aromatase.
Results: EGF was present in endometrial gland and lumenal epithelia from day 20 but staining intensity increased noticeably for the protein between days 30 and 40, coincidentally with the commencing secretion of equine Chorionic Gonadotrophin (eCG) from the endometrial cups and immediately prior to attachment and commencing interdigitation between the allantochorion and endometrium. EGF-R, on the other hand, was expressed strongly on the cell surface membrane of both non-invasive and invasive trophoblast and it similarly increased in staining intensity between days 30 and 40. Aromatase, the enzyme necessary for conversion of C-19 androgens to C-18 oestrogens, was expressed strongly and constantly from as early as day 12 in the non-invasive trophoblast of the allantochorion, but not the invasive trophoblast of the chorionic girdle, the progenitor tissue of the endometrial cups.
Discussion: The findings support the hypothesis that, in equine pregnancy, the maternal growth factor EGF synergises with maternally and fetally secreted oestrogens to drive the rapid growth and extensive vascularisation of the non-invasive, epitheliochorial, microcotyledonary placenta which results in the birth of the precocious foal after only 11 months gestation.

1. Introduction

Previous studies have demonstrated that Epidermal Growth Factor (EGF) and its receptor (EGF-R) play important roles in initiating placentation and development of both maternal and fetal interface tissues throughout gestation in women [1] and a variety of farm animal species including the sheep [2–4], goat [5], pig [6,7] and cow [8,9].

Likewise, in the pregnant mare, northern blot and *in situ* hybridisation techniques demonstrated a marked increase in EGFmRNA in the endometrium around day 38 after ovulation, just prior to attachment of the trophoblast of the allantochorion to the luminal epithelium of the endometrium and the ensuing interdigitation of maternal and fetal tissues. Expression remained elevated until at least day 250 of gestation and ^{125}I-labelled EGF binding detected EGF-R in both the allantochorion and the endometrium during the same period of pregnancy [10–12]. Curiously, and in contrast to the situation in other species where oestrogen has been shown to stimulate the expression of EGF and EGF-R [13–16], experiments involving the administration of exogenous progesterone and/or oestrogen to anoestrous and ovariectomised mares revealed that EGFmRNA could be upregulated in the epithelium of the endometrial glands by progesterone alone, provided it was administered for 35 consecutive days [17]. This finding supported the demonstration by Lennard et al. [12] of the upregulation of EGFmRNA in the endometrial glands around day 38 of gestation.

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The acquisition of 11 pregnant horse uteri perfused-fixed between days 20 and 68 of gestation, together with good, cross-reactive antisera raised against human EGF and EGF-R, and aromatase, the steroid enzyme necessary for conversion of androgens to oestrogens, initiated the present immunohistochemical experiments to monitor immunoreactive EGF, EGFR and aromatase at the placental interface around the time of implantation and placentation. Particular attention was paid to the periods before, during and after invasion of the endometrium by the specialised trophoblast cells of the chorionic girdle to form the equine Chorionic Gonadotrophin (eCG)-secreting endometrial cups and the immediately ensuing microvillous attachment and complex interdigitation between the non-invasive trophoblast of the allantochorion and the luminal epithelium (LE) of the endometrium to form the diffuse, non-invasive, epitheliochorial placenta [18].

2. Materials and methods

2.1. Tissues

Eleven known-stage gravid horse uteri in normal, healthy, young mares monitored ultrasonographically once weekly from day 14 after ovulation before they were killed by free bullet on days 20, 24, 26, 29, 32, 34, 37, 40, 46, 58 and 68 after ovulation were recovered intact within 30 min after death and perfuse-fixed with 4% w:v paraformaldehyde solution as described previously [19].

The Equine Fertility Unit in Newmarket where the mares were maintained was licenced under, and the experiment was approved by and carried out in accordance with, the UK Animals (Scientific Procedures) Act 1986.

The uterine wall and endometrium were dissected away from one third of the ventral surface of the conceptus bulge to expose the undisturbed conceptus *in situ*. This enabled pieces of endometrium with overlying choriovitelline or chorioallantoic membranes to be recovered from interesting parts of the conceptus and with minimal disturbance of the physical relationship between maternal and fetal tissues. Multiple paraffin-embedded blocks were made from each uterus from which multiple, sometimes serial, 5 μm sections were cut to best exhibit the most representative arrangement of membranes and endometrium. Sections were also cut from archived paraffin-embedded blocks of dioestrous endometrium, a day 309 placenta and the membranes of day 12 and day 14 conceptuses.

2.2. Immunohistochemical staining

A rabbit monoclonal antibody generated against recombinant human EGF (EGF [701538]; Life Technologies, Frederick, MD 21704, USA) and a rabbit polyclonal antiserum raised against human EGF-R (EGF-R [1005]: sc-03; Santa Cruz Biotechnology Inc., CA, USA), both reported to react with equine EGF and EGF-R by the manufacturers, and a mouse monoclonal generated against a synthetic peptide of human cytochrome P450 aromatase (MAC2077S; AbD Serotec, NC, USA), were used as primary antibodies at dilutions of 1: 50, 1: 25 and 1:5000 respectively. The sections were placed in a 56 °C oven overnight to de-wax them after which they were transferred to a Dako Plus Autostainer (Dako UK Ltd.) in which computer controlled indirect staining was carried out as described previously [20].

At least 5 sections cut at different levels through each block were stained with each of the 3 antibodies and these were examined and photographed by the same individual using an Olympus Laborlux microscope with an inbuilt camera. Care was taken to examine the stained sections sequentially with respect to increasing gestational age and thereby be able to assess increases or decreases in staining intensity that may have occurred with advancing gestation. Negative controls were run on the placental interface tissues by replacing the primary antiserum with appropriately diluted non-immune mouse and rabbit sera.

3. Results

3.1. EGF and EGFR

Labelling the horse tissues with the anti-human EGF and EGF-R sera gave even and precise staining patterns, the intensity of which for both antibodies increased subjectively with advancing gestation (Figs. 1 and 2). In dioestrus, the glandular epithelial cells showed definite staining for EGF, with almost no staining of the lumenal epithelium (Fig. 1a). At day 20 of gestation, staining intensity in the gland epithelium had increased markedly but was still lighter and more patchy in both the lumenal epithelium and the trophoblast (Fig. 1b). With the EGF-R antiserum, on the other hand, dioestrous endometrium remained unstained and, at day 20 of gestation, only the epithelium of the apical endometrial glands and the trophoblast of the choriovitelline membrane showed weak staining concentrated towards the surface of the trophoblast cells. The blastocyst capsule did not stain with either antibody (Fig. 2a).

Intensity of staining for EGF increased steadily in the luminal and endometrial gland epithelia and in the trophoblast between days 20 and 29 (Fig. 1c). EGF-R staining during the same period remained light and patchy in the luminal and gland epithelia on the maternal side of the interface but increased markedly in the trophoblast layer and now became concentrated on the cell surface membranes (Fig. 2b). Between days 30 and 40 EGF staining increased markedly in the cytoplasm of the epithelial cells lining the apical portions of the endometrial glands and to a lesser extent in the luminal epithelium and overlying trophoblast. The chorionic girdle cells also showed moderate staining for EGF at days 32 and 34 and this continued at day 37 when the girdle cells had begun to invade the maternal endometrium (Fig. 1d) and transform into mature, binucleate, eCG-secreting endometrial cup cells. Staining intensity in the endometrial gland epithelium remained high except in the distended basal portion of the glands within the endometrial cup and in glands distended as a result of age-related degeneration and 'walling off' by the concentric deposition of fibrous tissue ("gland nests"; Fig. 1c).

Staining for EGF-R during days 30–40 showed an even more dramatic upregulation in intensity on the trophoblast, especially the invasive trophoblast of the chorionic girdle where the individual girdle cells were sharply outlined by very precise and intense staining of their lateral and apical surfaces (Fig. 2c); this surface staining of the girdle cells continued unabated after they had invaded the endometrium and transformed into large endometrial cup cells (Fig. 2d). The EGF-R antibody also stained strongly the newly generated luminal epithelium covering the surface of the young endometrial cups but showed only pale staining of the endometrial gland epithelium (Fig. 2d).

Between days 40 and 68 as attachment and interdigitation of the allantochorion and endometrium was taking place, the EGF serum continued to show strong, even staining of the cytoplasm of the gland epithelial cells, with more moderate staining of the now intimately associated luminal epithelium and trophoblast (Fig. 1f); the histotroph within the endometrial glands also stained positively for EGF throughout the period (Fig. 1b, d and f). The EGF-R antiserum continued to stain the surfaces of the trophoblast cells, both invasive and non-invasive, strongly between days 40 and 68 (Fig. 2e& f). Staining was particularly intense on the unattached, pseudostratified columnar trophoblast cells in the lateral regions of each areolus (Fig. 2e and f) and was still localised to the cell surface (Fig. 2f). The epithelium of the endometrial glands also now stained