Localization and characterization of EGF/TGF- α receptors on peri-implantation trophoblast in sheep

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Receptors for epidermal growth factor (EGF) have been identified on the ovine trophoblast as early as day 15 of gestation. A radioligand assay with ^{125}I -labelled EGF was used to detect high and low affinity binding sites on the trophoblastic and placental membranes. The binding of ^{125}I -labelled EGF was inhibited by increasing concentrations of unlabelled EGF. Competition studies with other peptide hormones including transforming growth factor α (TGF- α), insulin-like growth factor-I (IGF-I) and ovine placental lactogen confirmed the specificity of EGF/TGF- α for its receptor. Cross-linking experiments using disuccinimidyl suberate (DSS) revealed a radiolabelled band of relative molecular mass 170 kDa. Immunohistochemical localization of the receptors demonstrated their distribution on the epithelial layer cells. The presence of receptors for EGF/TGF- α suggests that these factors could be involved in the regulation of embryonic development and fetal growth.

Introduction

Epidermal growth factor (EGF) was first isolated from mouse submaxillary gland (Cohen, 1962) and subsequently from various other organs and body fluids of mammals. EGF stimulates the growth of cells *in vivo* (Cohen and Elliot, 1963; Cohen, 1965; Carpenter and Cohen, 1979; Gospodarowicz, 1981) and *in vitro* (Sun and Green, 1977; Gospodarowicz *et al.*, 1978; Carpenter and Cohen, 1979; Adamson and Rees, 1981; Hata *et al.*, 1990). Through its ability to act as a mitogen, EGF may play a role in fetal and placental growth (O'Keefe *et al.*, 1974; Adamson *et al.*, 1981; Thorburn *et al.*, 1981). In fact, EGF stimulates the proliferation of embryonic cells both in monolayer cultures and during embryonic morphogenesis *in vivo* and *in vitro* (Sundell *et al.*, 1975; Goldin and Opperman, 1980; Pratt, 1980; Yoneda and Pratt, 1981; Kaplowitz *et al.*, 1982; Gross *et al.*, 1986).

The physiological actions of EGF result from its binding to specific receptors present on the outer cell membranes of target cells. The EGF receptor is a glycoprotein with tyrosine kinase activity (Cohen and Carpenter, 1975; Schlessinger *et al.*, 1983). This receptor has been detected in numerous tissues, including the uterus (Hofmann *et al.*, 1984) and the placenta (Hock and Hollenberg, 1980; Carson *et al.*, 1983; Lai *et al.*, 1986). However, despite the wealth of literature on EGF and its receptor in placental function, we are not aware of any studies that describe the presence of the EGF receptor in the preimplantation ovine trophoblast, as the embryo prepares to adhere to the uterine epithelium. Moreover, at this time, the conceptus undergoes rapid growth and development. The factors that control cell proliferation and remodelling in the developing blastocyst are not well known, but EGF may be involved in these processes.

Uterine tissues from several species contain various polypeptide mitogens, including EGF (Brigstock *et al.*, 1989).

In the present study, we have investigated and characterized the binding sites for EGF on the ovine trophoblast at the implantation period. In comparison, we also used third-term ovine placentae, which express a high number of EGF/TGF- α receptors on the microvillus plasma membranes.

Materials and Methods

Animals and tissues

Oestrus was synchronized in Préalpes du Sud ewes with intravaginal sponges (40 mg, 17 α -acetoxy-9 α -fluoro-11 β -hydroxyprogesterone; Intervet, France), which were removed after 14 days. Ewes then received one i.m. injection of 500 iu PMSG (Intervet, France). On the day of oestrus, which occurred 48 h later and was designated as day 0, the ewes were mated twice. Ewes were killed on days 15, 30 and 120 of gestation. The reproductive tract was removed. Conceptuses (day 15) were collected by flushing the uterine horns with sterile 0.1 mol PBS l $^{-1}$, pH 7.2, or recovered by incision of the uterus (days 30 and 120). The trophoblast was separated from the embryo, yolk sac and allantoic membranes. From day 120, only fetal cotyledons were used for experiments.

Tissues were used either fresh or frozen in liquid nitrogen and stored at -70°C until required.

Growth factors and peptides

Mouse epidermal growth factor (Receptor grade) from Genzyme Corporation, UK; transforming growth factor- α , human recombinant from Promega Corporation, USA; insulin-like

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growth factor-I, recombinant analogue of human somatomedin C from Amersham International plc, UK were used in this study. The ovine placental lactogen was purified in our laboratory (Martal, 1978). Mouse EGF was iodinated, using the method of Greenwood *et al.* (1963). In these conditions, the specific activity of ^{125}I -labelled EGF obtained was from 70 to 200 $\mu\text{Ci } \mu\text{g}^{-1}$.

Membrane preparation

Membranes were prepared from ovine extraembryonic tissues (trophoblast, fetal cotyledons) using the procedure of Shiu *et al.* (1973) as modified by Martal and Djiane (1977). The following steps were carried out at 4°C . The tissue was cut into small pieces and homogenized with an ultra-turrax homogenizer, in 0.3 mol sucrose l^{-1} . The homogenate was filtered through two layers of cheese-cloth and centrifuged at 15 000 g for 20 min to isolate intracellular organelles. The supernatant was then ultra-centrifuged at 105 000 g for 90 min. The pellet was washed twice and resuspended, using a glass homogenizer, in 25 mmol Tris-HCl buffer l^{-1} (pH 7.5), 10 mmol MgCl_2 l^{-1} . The protein concentration was determined by the method of Lowry *et al.* (1951), using BSA as a standard. Preliminary studies indicated that membrane preparations could be stored for more than six months at -20°C without change in its ability to bind EGF.

Radioligand assay

The assay was performed on membranes from the three different stages of pregnancy. To each assay tube was added successively 200 μl of assay buffer (25 mmol Tris-HCl l^{-1} pH 7.5, 10 mmol MgCl_2 l^{-1} 0.1% (w/v) BSA: RIA grade – Sigma, France), 100 μl of mouse EGF (1–200 ng ml^{-1}), 100 μl of ^{125}I -labelled mouse EGF (100 000 c.p.m.) and ovine membranes (100 μl containing 250 μg of proteins). The binding reaction was performed at room temperature for 3 h and was stopped by the addition of 1.5 ml assay buffer to each tube. The membranes were collected by centrifugation at 2500 g for 30 min, at 4°C . The supernatant was discarded and the amount of radioactivity in the pellet measured on an LKB gamma counter. In some experiments, membrane preparations were incubated for various periods. The effect of increasing concentrations of membrane proteins on the binding efficiency was also examined.

The results were expressed as specific binding: difference between total and nonspecific binding, which was carried out in the presence of 1000-fold excess concentration of unlabelled EGF.

Saturation analysis

Saturation analysis was performed over a range of 0.04–3.20 nmol ^{125}I -labelled EGF l^{-1} . Nonspecific binding at each concentration of radiolabelled EGF was determined in the presence of unlabelled EGF at 1000-fold excess concentration.

Competition studies

Specificity of EGF binding was checked in the presence of the following competitors: TGF- α , IGF-I and ovine placental lactogen. The radioligand exchange assay was done, but with the substitution of the various peptides for unlabelled EGF.

Determination of molecular mass of EGF-binding sites

The membranes were incubated with ^{125}I -labelled EGF, in the absence or presence of unlabelled EGF, at pH 7.5, for 3 h at room temperature. At the end of incubation, 1.5 ml ice-cold binding buffer was added and the membranes centrifuged as described in the radioreceptor assay. The pellet was then washed twice with ice-cold PBS buffer, and subjected to cross-linking in the presence of 0.3 mmol disuccinimidyl suberate l^{-1} for 15 min at room temperature. The cross-linking agent was dissolved in dimethyl sulfoxide, immediately before use, and added to the membrane suspension to give a final concentration of 3% dimethyl sulfoxide in the binding buffer. The reaction was terminated by rapid centrifugation and washing of the membranes at 4°C , with excess binding buffer. The membrane pellets were solubilized in 100 μl of electrophoresis sample buffer containing 50 mmol Tris-HCl l^{-1} pH 6.8, 4% (w/v) sodium dodecyl sulfate (SDS), 6% (w/v) sucrose and 2% (v/v) bromophenol blue, in the presence of 50 mmol dithiothreitol l^{-1} . Samples were immediately heated to 100°C for 5 min, rapidly centrifuged (2500 g for 10 min) and analysed by one-dimensional SDS PAGE using 7.5% acrylamide in the separating gel, according to the method of Laemmli (1970). After electrophoresis, the gels were stained, destained, dried and exposed to Hyperfilm B_{max} (Amersham International plc) for 2–3 weeks at -70°C , with intensifying screens.

Immunohistofluorescence

Rabbit polyclonal antibody (Ab-4) against human EGF receptor (residues 1005–1016) was obtained from Oncogene Science Inc, USA. The tissues were fixed by immersion in 4% paraformaldehyde and embedded in paraffin. The sections (5 μm) were deparaffinized in xylene and treated with 0.05% saponin for 30 min at room temperature. The sections were incubated with 10% goat serum in PBS for 1 h to block non-specific binding of IgG. The antibody Ab-4 was used at a 1:10 dilution of the stock solution in PBS–2% BSA. The incubation was carried out either for 2 h at room temperature or overnight at 4°C . After three washes in PBS–2% BSA (10 min each), the sections were incubated with 1:200 goat antirabbit IgG–FITC (Pasteur Institute, France). As negative control, sections were incubated in nonimmune rabbit serum. After mounting, the slides were stored in the dark at 4°C until observations using a fluorescence microscope.

Statistical analysis

Specific binding data was analysed by a ligand computer program (Munson and Robard, 1980) to yield the dissociation constant (K_d) and the number of specific binding sites.

One way analyses of variance followed by Student–Newman–Keuls tests were used for statistical comparison.

Results

The optimal assay conditions for ^{125}I -labelled EGF binding to trophoblastic or placental membranes were first defined for

Fig. 1. (a) Time course of specific binding of ¹²⁵I-labelled epidermal growth factor (EGF) to ovine 15-day-old trophoblast membranes, at 4°C (○) and 22°C (■). Dissociation of ¹²⁵I-labelled EGF (---) from trophoblastic membranes can be obtained by addition of unlabelled EGF (500 ng) after equilibrium is reached. (b) Effect of increasing concentration of protein on the specific binding of ¹²⁵I-labelled EGF to ovine trophoblastic membrane preparation from day 15 of pregnancy. Data are the means of duplicate incubations in a representative experiment.

Fig. 2. Saturation curve of ¹²⁵I-labelled epidermal growth factor specific binding to ovine trophoblast membranes of a typical experiment.

further characterization of binding sites. Although significant specific binding of ¹²⁵I-labelled EGF to trophoblast membranes was obtained at 4°C, maximal specific binding was attained at

Fig. 3. Ligand specificity of the ovine trophoblast binding sites for epidermal growth factor (EGF). Each point is the mean of duplicate incubations in a representative experiment performed by addition of increasing concentrations of EGF (■), TGF- α (▲), IGF-I (△) and ovine placental lactogen (○) to trophoblastic membranes incubated with ¹²⁵I-labelled EGF.

Fig. 4. Scatchard analysis of specific binding of the ¹²⁵I-labelled epidermal growth factor (EGF) to ovine trophoblastic and placental membranes. ¹²⁵I-labelled EGF (0.08–0.24 pmol) was incubated with 250 μ g membrane protein for 3 h at 22°C. Each curve corresponds to the pooled data at (a) 15, (b) 30, and (c) 120 days of gestation.

Table 1. Ontogeny of the high- and low-affinity epidermal growth factor receptors during pregnancy in sheep

Days of pregnancy	High-affinity receptors		Low-affinity receptors	
	K_d (10^{-11} mol l $^{-1}$)	fmol mg $^{-1}$ tissue	K_d (10^{-8} mol l $^{-1}$)	fmol mg $^{-1}$ tissue
15 ($n = 6$)	4.11 ± 1.16	76.4 ± 2.3	3.54 ± 0.43	$2364 \pm 267^{**}$
30 ($n = 6$)	4.97 ± 0.28	75.0 ± 1.6	0.59 ± 0.13	$326 \pm 92^*$
120 ($n = 4$)	7.30 ± 3.56	71.3 ± 7.2	0.38 ± 0.15	$756 \pm 250^*$

The values represent means $\pm$ SEM.

** Significantly different from other groups ($P < 0.01$).

* Significantly different between the two values ($P < 0.05$).

Fig. 5. (a) Displacement curve and (b) Scatchard plot of specific binding of 125 I-labelled epidermal growth factor to day 15 trophoblast membranes which present only one class of binding sites, in three experiments. T: total radioactivity.

22°C, after 3 h of incubation (Fig. 1a). In addition, over 80% of this binding was reached from the first hour. Thus, binding was clearly dependent on time and temperature. 125 I-labelled EGF binding to membranes was reversible. The addition of unlabelled ligand (100 μ l, 5 μ g ml $^{-1}$) at the end of incubation immediately displaced the specific binding from 19.2 to 14.2%. This value decreased to 1.6% in 2 h. The optimal membrane protein concentration for maximal specific binding was determined by incubating with various amounts of protein. The specific binding of 125 I-labelled EGF increased in a linear fashion as a function of protein concentration up to 4 mg ml $^{-1}$, and there was no further significant increase in EGF binding at higher protein concentrations (Fig. 1b). On the basis of these data, all subsequent binding experiments were performed at 22°–25°C for 3 h, but with regard to the small amount of trophoblastic tissue at day 15 of pregnancy, a protein concentration of 2.5 mg ml $^{-1}$ was chosen for the routine binding assay.

Incubation of the membrane fractions with increasing concentration of 125 I-labelled EGF produced a saturation of the

binding protein at 0.8 nmol l $^{-1}$ (Fig. 2). The binding of 125 I-labelled EGF was displaced by unlabelled EGF and TGF- α in a dose-dependent manner (Fig. 3), but not by any other growth factor or hormone used (IGF-I, ovine placental lactogen), at a 1000-fold excess of the concentration of labelled EGF. More than 90% of 125 I-labelled EGF bound to membranes could be displaced by the highest concentration (10 μ g ml $^{-1}$) of EGF or TGF- α indicating that nonspecific binding, which does not change with the age of tissue, contributed to less than 10% of the total binding of EGF to trophoblast membranes.

At the three gestational ages studied, Scatchard analysis resulted in curvilinear plots indicating the presence of two classes of receptor (Fig. 4), with estimated dissociation constants of approximately 10^{-11} and 10^{-8} mol l $^{-1}$ for the high- and low-affinity receptors, respectively. The ontogeny of EGF binding sites present on the trophoblast is given (Table 1). Affinity of EGF to its receptor was similar at various gestational ages studied. The majority of the 125 I-labelled EGF binding was to the lower-affinity receptor class in which variations according to stage of pregnancy could be observed. The number of low-affinity receptors was very important in early pregnancy, decreased to a low value after implantation and a small increase could be observed at late gestation. In contrast, there was no change in the number of high-affinity receptors. Nevertheless, at day 15 of gestation, 33% of the cases examined ($n = 9$) showed only high-affinity receptors (Fig. 5).

The autoradiograms of SDS-PAGE revealed a single band, corresponding to EGF-receptor with a relative molecular mass of 170 kDa (Fig. 6). Specificity of binding was evident since it was displaced progressively by increasing doses of unlabelled EGF. Similar results were obtained with TGF- α (data not shown).

The immunohistochemical studies showed positive staining on the trophectoderm cells (Fig. 7a). A typical control obtained by using normal rabbit serum shows only background fluorescence (Fig. 7b).

Discussion

Most of the experiments investigating the role of EGF in mammalian embryogenesis have been developed in humans and rodents, especially mice. With few exceptions, pig (Corps *et al.*,

Fig. 6. Autoradiographic profile of ^{125}I -labelled epidermal growth factor (EGF) binding to specific ovine trophoblastic and placental receptors in absence or presence of 500 ng (+) and 1 μg + of unlabelled EGF at days 15, 30 and 120 of gestation. High molecular weight markers used were: myosin, β galactosidase, phosphorylase b, BSA and ovalbumin.

1990) and cows (Watson *et al.*, 1992), there is no more information on farm animals. In this study, EGF receptors were shown to be present on ovine trophoblast as early as day 15 of pregnancy, which corresponds to the beginning of implantation in this species. Similar results have been obtained in pigs in which ^{125}I -labelled EGF bound to trophoderm cells from 15- to 19-day-old blastocysts (Corps *et al.*, 1990).

Because both EGF and TGF- α bound with the same affinity to EGF receptors, these EGF binding sites on ovine trophoblast may represent receptors for EGF or TGF- α . The rapid rate of association of labelled ligand with the receptor site agrees with the data of O'Keefe *et al.* (1974) and Carpenter *et al.* (1975). The binding is specific, saturable and reversible. Incomplete dissociation following addition of excess unlabelled EGF may be due to the formation of non-dissociable covalent binding of ^{125}I -labelled EGF to some receptor sites. The formation of this covalent binding may have resulted from the use of chloramine T in the radioiodination procedure (Comens *et al.*, 1982; Wynn *et al.*, 1989). In the study reported here, EGF receptor concentration varies with gestational age and the highest concentration appears in early pregnancy (Chegini and Rao, 1985; Marou *et al.*, 1987). The preimplantation period in sheep is a time of extensive cellular remodelling during which the day-11 spherical blastocyst of 1 mm diameter extends to a filamentous thread up to 10 cm in length in 3–4 days. Damjanov *et al.* (1984) showed that proliferative epithelial cells have a relatively large number of EGF receptors, but after cell differentiation and arrest of proliferation, the number of EGF receptors is reduced to low values. This finding suggests that the factor might play a role in regulating trophoblast growth and embryo development.

Scatchard analysis discloses the possibility of two classes of receptor: a major class of low-affinity and a minor class of high-affinity EGF receptors. High- and low-affinity sites have been described previously in the human placenta (O'Keefe *et al.*, 1974; Carson *et al.*, 1983) and in mouse placenta (Smith and Talamantes, 1986). However, only one type of receptor was described in mouse embryonic tissues, including placenta by Adamson and Meek (1984). The functional implications of the existence of high-affinity and low-affinity EGF receptors are largely obscure. The high-affinity EGF receptors have been implicated as the subclass through which EGF predominantly exerts its mitogenic effect. A correlation between the relative number of high-affinity EGF receptors and the activation of EGF receptor kinase has been established (Defize *et al.*, 1989). However, it seems that a negative cooperativity was exerted by this growth factor. In a great variety of cells, EGF reduced the affinity of its own receptors, which undergo a ligand-dependent transition from a homogeneous (high-affinity) population to a heterogeneous population and finally to a homogeneous (low-affinity) population (Wiley *et al.*, 1989). Cross-linking studies showed a molecular mass of 170 kDa in agreement with previous characterization by Carpenter (1987). The intensity of autoradiographic bands is greatest at day 15 of pregnancy, indicating a very large number of receptors at this stage. Immunohistological observations confirmed the presence of EGF receptors in ovine trophoblastic tissue, with a localization on ectodermal cells.

We attempted to measure EGF in sheep using the human EGF radioimmunoassay and two radioreceptor assays using a suspension of membranes of human A431 cells and sheep

Fig. 7. Immunohistochemical staining of the day 15 ovine trophoblast using the anti-human epidermal growth factor receptor rabbit antibody (Ab-4). (a) Intense immunoreactivity of EGF receptors on the trophoderm cells ($\times 400$); (b) Negative control serum ($\times 320$).

placental membranes. With these quantitative methods, we could not detect significant amounts of EGF in culture media or extracts of ovine conceptus from days 12 to 30 of gestation. EGF was not detected in allantoic or amniotic fluids, although EGF could be detected by radioreceptor assay in saliva, urine and milk (Gharib-Hamrouche, unpublished data). Our inability to detect any ovine embryonic EGF or EGF-like activity may be due to the inherent limitations of the assay methods that were used. The radioimmunoassay could measure concentrations in the pg ml^{-1} range, but is hampered by high specificity. For

example, despite the high degree of sequence similarity between human and mouse EGF, antisera to human EGF do not crossreact with mouse EGF. A similar lack of immunological crossreactivity with the ovine EGF has been reported by Thorburn *et al.* (1981) and may explain the inability to detect any ovine EGF using antisera to human EGF. Radioreceptor assays display less species specificity than radioimmunoassays, but the levels of ovine EGF activity, like other growth factors mediating cell-cell interactions, are below the limit of sensitivity of the assay (0.1 ng ml^{-1}). The nature and source of the

physiologically relevant ligand therefore remains to be established. In late pregnancy of humans, Eaton *et al.* (1987) found that only very small amounts of intact ^{125}I -labelled EGF (0.2% of that added to maternal side) were transported from the maternal to the fetal circulation. However, the possibility that EGF is provided from the plasma of the mother cannot be completely discounted. The uterine luminal fluid has been shown to contain molecules with growth promoting activity for cells in culture: IGF-I, heparin-binding growth factors and a novel factor that has been termed uterine luminal fluid mitogen (Simmen *et al.*, 1989). Most of the polypeptides found in the luminal uterine fluid derived from the plasma by transudation (Roberts and Parker, 1974; Roberts *et al.*, 1976). A further possibility has been proposed by Nexø *et al.* (1980) and Popliker *et al.* (1987). With regard to the presence of EGF receptor mRNA in fetal mouse but low amounts or absence of EGF and EGF precursor mRNA in most fetal rodent tissues, it is possible that the ligand for embryonic or fetal receptors in mice is TGF- α rather than EGF. In fact, it is well known that EGF and TGF- α bind to the same cell surface receptors and compete with each other for binding sites. These two growth factors have similar actions, but there are distinguishable differences in their biological activity. Rappolee *et al.* (1988) and Werb (1990) detected TGF- α mRNA sequences and peptides in preimplantation mouse development. However, Han *et al.* (1987) showed that TGF- α mRNA is expressed in the decidual cells, which also present EGF/TGF- α binding sites. Similar data have been obtained for the sheep fetus by Freemark and Comer (1978).

Thus, TGF- α via its receptor, may have an autocrine or paracrine function or both functions on decidual growth and could probably take part in the process of implantation and in the trophoblast elongation or embryo development.

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