Flow cytometric detection and binding studies of human endometrial stromal cell epidermal growth factor receptor in monolayer culture: influence of progesterone

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Epidermal growth factor (EGF) has been shown to modulate endometrial differentiation in vivo and in vitro. Therefore, endometrial stromal cell EGF receptors were characterized in intact endometrial stromal cells, cultured in vitro. The methods used for characterization were flow cytometry, binding and displacement studies, and gel electrophoresis followed by autoradiography. In flow cytometry the histogram of labelled stromal cells was identical to Caski cells, which served as a positive control. EGF binding revealed the typical binding hierarchy for EGF receptors. The Scatchard analysis showed a curvilinear plot with a calculated dissociation constant of 0.36 nM for high affinity binding sites. In autoradiography a band of ~170 kDa was visualized corresponding to the known size of the EGF receptor. The intensity of this band was increased by pretreatment of stromal cells with 10 nM progesterone for 4 days. Furthermore, stimulation with progesterone led to an increase in specific EGF binding activity of stromal cells by 21% compared to control. These data indicate that intact stromal cells in monolayer culture maintain specific EGF receptors, which are up-regulated by progesterone.

Key words: EGF receptor/EGF/endometrium/stromal cell culture

Introduction

Human endometrial differentiation is controlled by steroid sex hormones, oestrogen and progesterone. However, the hormonal action might be transmitted by several growth factors, for example the epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I), in an auto/paracrine manner (Giudice, 1994; Smith, 1994).

EGF has been shown to modulate endometrial differentiation in vivo and in vitro. In ovariectomized mice EGF mimicks oestrogen effects on vaginal and uterine cellular proliferation (Nelson et al., 1991) and may induce decidualization in rats (Tamada et al., 1994). Furthermore, EGF displays mitogenic activity in endometrial tissues (DiAugustine et al., 1988). The EGF peptide itself is predominantly expressed in the stromal cellular compartment (Berchuck et al., 1989; Hofmann et al., 1991). Since the stromal cell is the target for decidualization, EGF is of particular interest for the decidualization process. In vitro data provide strong evidence that EGF in the presence of serum may contribute to the decidualization process (Irwin et al., 1989), interacting with other serum factors such as platelet-derived growth factor (PDGF) (Tang et al., 1994). Furthermore EGF might contribute to the proliferation of endometrial stromal cells in vitro in serum-supplemented media (Mellor and Thomas, 1995).

Binding sites of EGF in human endometrium have been described (Hofmann et al., 1984) and specific EGF receptors are distributed in the human endometrium throughout the menstrual cycle (Damjanov et al., 1986; Berchuck et al., 1989; Smith et al., 1991; Bigsby et al., 1992; Chegini et al., 1992; Prentice et al., 1992), predominantly in the stromal cell layer. Although some authors failed to demonstrate a cycle-dependent staining for EGF receptors (Damjanov et al., 1986; Berchuck et al., 1989), binding studies of endometrial samples of different cycle phases revealed maximum EGF binding in the midcycle phase (Taketani and Mizuno, 1988). Concomitantly, the highest mRNA EGF receptor levels were detected in the midproliferative phase (Heiner et al., 1993).

In mixed endometrial cell cultures, it has been shown that EGF-binding activity increases in the presence of progesterone, giving further evidence that EGF and progesterone might both contribute to decidualizing effects (Taketani and Mizuno, 1991; Watson et al., 1994). Since stromal cells are the target compartment of decidualization we have established pure stromal cell monolayer cultures and identified the nature of EGF binding component in intact monolayers by binding studies and flow cytometry. Furthermore we have shown that monolayer cultures are a suitable system to study interactions of progesterone and EGF by demonstrating an altered binding pattern of EGF to its nominative receptor in a progesterone-dominated milieu of intact cellular layers.

Materials and methods

The culture medium, minimal essential medium (MEM) with L-glutamine, fetal calf serum (FCS), and trypsin–EDTA solution (Puck’s solution) were obtained from Gibco (Eggenstein, Germany). Imipenem and nystatin were purchased from Lederle Arzneimittel (Wolfratshausen, Germany). Progesterone, collagenase type 1, phenylmethylsulphonyl fluoride, benzamidine, bacitracin and aprotinin were obtained from...
Sigma Chemical Co. (Munich, Germany). Unlabelled EGF, fibroblast growth factor (FGF) and transforming growth factor-α (TGFα) were from Boehringer Mannheim (Mannheim, Germany); \(^{125}\text{I}\)EGF was from Amersham (Braunschweig, Germany; specific activity 100 μCi/μg). Acrylamide, N,N-methylene bisacrylamide, N,N,N, N-tetramethyl-ethylenediamine and disuccinimidyl suberate (DSS) were from ICN Biomedical Inc. (Cleveland, OH, USA).

Endometrial stromal cell culture

Endometrial samples were obtained from hysterectomy specimens in the proliferative phase of the menstrual cycle, after the patient’s informed consent. Stromal cells were isolated from epithelial cells as described previously with slight modification by enzymatic digestion with collagenase and filtration through a 50 μm nylon sieve (Satyaswaroop et al., 1979; Singer and Strowitzki, 1996). The filtered cell suspension was then seeded in a 75 ml culture flask containing 15 ml MEM with L-glutamine supplemented with 10% FCS, 10% calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamycin and 0.25 μg/ml EDTA (Gibco, Eggenstein, Germany). After 48 h at 37°C, 1 ml medium was removed, and the monolayer was washed twice with 3 ml 4°C binding buffer and then incubated with 1.5 ml binding buffer [PBS + 0.1% bovine serum albumin (BSA), pH 7.4] containing 75 000 c.p.m. labelled \(^{125}\text{I}\)EGF in the absence and presence of 1 μg unlabelled EGF at 4°C and 25°C for various time intervals. After this incubation, the binding buffer was aspirated and the monolayer was washed twice with 3 ml 4°C binding buffer, then incubated with 1.0 ml 0.5 N NaOH for 1 h at 37°C. The cell lysate was then transferred to a counting vial and every well was washed twice with 0.5 ml 0.5 N NaOH. The washing solution was pooled with the corresponding cell lysate and the radioactivity associated with the lysate was counted in a γ-spectrometer. Specific binding was determined by subtracting the radioactivity counted in an excess of unlabelled EGF from the total binding in the absence of unlabelled EGF.

Competitive binding studies

For competitive binding studies and displacement curves confluent monolayer cultures in multiwell plates (~200 000 cells per well) were incubated with 1.5 ml binding buffer containing 50 000 c.p.m. labelled EGF in the presence of serial concentrations (1, 2.5, 7.5 and 80 nM) of the unlabelled peptides EGF, FGF and TGFα for 2 h at 25°C. The unbound radioactive \(^{125}\text{I}\)EGF was removed by aspirating the binding buffer, the monolayer was washed twice with 3 ml 4°C binding buffer and then lysed by incubating with 1.0 ml 0.5 N NaOH for 1 h at 37°C. The cell lysate was transferred to counting vials, and every well was washed twice with 0.5 ml 0.5 N NaOH. The washing solution was pooled to the corresponding cell lysate. The radioactivity associated with the cell lysate was counted in a γ-spectrometer and expressed as percentage of binding to the radioactivity measured in the absence of unlabelled ligands. Scatchard analysis was performed using a computer program as described by McPherson (1985).

Effect of progesterone on \(^{125}\text{I}\) EGF binding to stromal monolayer cultures

Two 6-well plates (diameter 3.5 cm) were used in this study. A total of 200 000 cells/well were incubated with a medium containing 10 nM progesterone. Progesterone was dissolved in ethanol and added to the culture medium at a final concentration of 0.1% v/v. The control plate received a culture medium containing 0.1% v/v ethanol without progesterone. The monolayer was stimulated with progesterone for 4 days and the medium was changed on alternate days. After stimulation had been completed, the medium was removed and the monolayer was washed twice with 3 ml 4°C binding buffer and then incubated with 1.5 ml binding buffer containing 50 000 c.p.m. labelled EGF in the absence and presence of 1 μg unlabelled EGF at 25°C. The unbound radioactive EGF was separated by aspirating the binding buffer and washing the monolayer twice with 3 ml 4°C binding buffer. The monolayer was then lysed by incubation in 1.5 ml 0.5 N NaOH for 1 h at 37°C. The cell lysate was transferred to counting vials and the radioactivity associated with cells was counted in a γ-spectrometer. Specific binding was determined by subtracting the radioactivity measured in the presence of an excess unlabelled EGF from the total activity measured in absence of unlabelled EGF.

Affinity cross-linking and autoradiography

To determine the nature of the EGF-binding component of the stromal cell surface, an affinity cross-linking technique was used. Stromal cells (0.5×10⁶ cells) were plated in 10 cm Petri dishes and allowed to grow in 12 ml culture medium MEM supplemented with 10% FCS. At confluence, the medium was removed and the monolayers were washed twice with 15 ml cold PBS, pH 7.4 with gentle shaking for 10 min and then incubated with 10 ml binding buffer (PBS +
0.1% BSA, pH 7.4) containing 2×10^6 c.p.m. labelled [125I]EGF for 2 h at 25°C. The unbound radioactive EGF was removed by aspirating the binding buffer, the monolayer was then washed, incubated with 10 ml PBS, pH 7.4, containing freshly prepared DSS (7 mM in dimethyl sulphoxide) at a final concentration of 0.1 mM for 15 min at 25°C for the cross-linking reaction. The cross-linking reaction was then terminated by adding 2.5 ml 500 mM Tris–HCl/50 mM EDTA, pH 7.4. The cross-linking buffer was aspirated, the monolayer washed twice with 15 ml 100 mM Tris/10 mM EDTA, pH 7.4, scraped with a rubber policeman, and centrifuged for 15 min at 850 g at 25°C. The supernatant was discarded, the pellet was dissolved in 250 µl electrophoresis sample buffer containing 3% sodium dodecyl sulphate (SDS), 50 mM Tris/HCl, pH 7.0, 10% glycerol (w/v), 0.001% (w/v) bromophenol blue and 100 mM dithiothreitol as a reducing agent. The sample was then boiled for 5 min, centrifuged for 2–3 min at 500 g and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) on a 7.5% minigel slab according to Laemmli (1970). At the end of the electrophoresis, the gel was fixed in 40% methanol solution, stained with 0.25% Coomassie blue, destained with 30% methanol/ 10% acetic acid solution and then vacuum-dried. Finally the gel was subjected to autoradiography by exposing to Kodak X-OMAT AR film for 5–10 days at –20°C. The molecular weight of radiolabelled protein bands was estimated by comparison with the migration pattern of standard marker proteins.

All studies were performed with three different endometrial stromal cell cultures unless otherwise noted.

**Results**

**Flow cytometric analysis**

Flow cytometric analyses have been carried out using cell suspension. Caski cells, a cervical cancer cell line overexpressing EGF receptors, served as positive control. Caski cells showed an intense fluorescence due to specific EGF receptor staining when compared to lymphocytes (Figure 1). Endometrial stromal cells displayed a similar shift, indicating that endometrial stromal cells in vitro express specific EGF receptors. It should be noted that autofluorescence depends on cell size; Caski cells and stromal cells are of similar size. Moreover, a negative control with antibodies was run in each experiment (see Materials and methods).

**Determination of optimal time and temperature conditions of [125I]EGF binding to stromal cell monolayer cultures**

At 24°C maximum binding was obtained after 90 min (Figure 2). Binding was stable for at least a further 90 min without decline. Specific binding was ~3.5% of the total radioactive input. At 4°C a similar binding curve was obtained, but with less binding than at 24°C. Therefore all experiments were performed at 24°C, allowing stable binding conditions for at least 2 h.

**Competitive binding studies**

To determine the specificity of surface EGF binding activity of stromal cells, a displacement binding curve was constructed, where the stromal monolayer was incubated with radiolabelled [125I]EGF in presence of serial concentrations of the unlabelled ligands EGF, TGFα and FGF (Figure 3). Unlabelled EGF is the most potent competitor displacing the bound [125I]EGF from the cellular surface of stromal cells with half-maximum displacement at nearly 5 nM. TGFα was less effective in comparison to unlabelled FGF. Furthermore, FGF displacement displayed a linear relationship without any decrease in binding with increasing concentrations of unlabelled FGF, indicating a weak affinity and low specificity. Scatchard analysis of binding data showed a curvilinear plot, indicating the involvement of multiple binding components on the stromal cell surface that may partially interfere with EGF binding activity (Figure 4). The dissociation constant of EGF binding activity on the surface of stromal cells, calculated from Scatchard data, was 0.36 nM.

**Effect of progesterone on EGF binding activity of stromal cells in monolayer culture**

Variations of specific binding of labelled [125I]EGF to stromal cell monolayers under the influence of progesterone have been studied. The stimulation of stromal monolayers with 10 nM progesterone for 4 days led to an increase in specific EGF binding activity of stromal cells by 21% compared to the control (Figure 5). Cell numbers between both groups were identical.

**Affinity cross-linking and autoradiography**

To identify the nature and approximate molecular weight of the EGF binding component on the stromal cell surface, we have applied an affinity cross-linking technique. In Figure 6, a single radiolabelled protein band under reducing conditions with an apparent molecular weight of 170 kDa is visible. This band has the same migration pattern as EGF receptor species already identified in A431 cells (Buhrow et al., 1982). When the stromal cells in monolayer culture were pretreated with progesterone the intensity of the resulting band was greater than that of control, indicating that progesterone might increase the EGF receptor expression or the EGF binding activity of endometrial stromal cells. These findings confirm the already observed increase of EGF binding activity of stromal monolayers by progesterone.

**Discussion**

The decidualization changes of human endometrium occur under the influence of progesterone and oestrogens during the secretory phase (Giudice, 1994). Several growth factors including EGF, IGF-I, IGF-II and TGFβ have been identified in endometrial tissue (Giudice, 1994). The immunolocalization of the EGF peptide, and the detection of IGF-I and IGF-II mRNA throughout the menstrual cycle suggested that these factors are involved in endometrial cyclic activity (Hofmann et al., 1991). In particular, the role of the EGF in endometrial differentiation and decidualization has been emphasized in different studies in vivo and in vitro. The EGF receptor is predominantly expressed in the endometrial stromal compartment (Chegini et al., 1986) and shows a maximum level of expression in the pre-ovulatory phase of the menstrual cycle (Taketani and Mizuno, 1988; Bonaccorsi et al., 1989; Troche et al., 1991). In vitro studies have shown that complete decidualization in a culture of endometrial stromal cells
directly depends on the presence of progesterone and EGF (Irwin et al., 1989). Furthermore, EGF acts as a potent stimulus of stromal cell proliferation in vitro (Taketani et al., 1989; Irwin et al., 1991; Strowitzki et al., 1991; Mellor and Thomas, 1995) and modulates the secretion of plasminogen activator inhibitor-I in human endometrial stromal cells in vitro (Sandberg et al., 1997). Therefore, we have applied flow cytometric analysis as a new technique in combination with affinity cross-linking to demonstrate the existence of EGF surface receptors in monolayer cultures of endometrial stromal cells as an in vitro model. Although we have characterized EGF receptors in endometrial stromal membrane preparations in a previous study by binding studies and autoradiography (Singer and Strowitzki, 1996), no direct information on the expression of EGF receptors in intact cells in monolayer cultures resulted from these studies. We therefore have conducted this study in intact monolayers of human endometrial stroma in order to investigate the suitability of stromal cell cultures for EGF receptor studies under in vitro conditions.

Flow cytometry is particularly suitable for studying intact cells in suspension. We have used Caski cells, an epidermoid cancer cell line, as a reference due to its high content of
Figure 4. Scatchard analysis of [$^{125}$I]epidermal growth factor (EGF) binding data of intact endometrial stromal cells; the dissociation constant was 0.36 nM.

Figure 5. Specific binding of [$^{125}$I]epidermal growth factor (EGF) to intact endometrial stromal cells with and without stimulation of monolayer cultures with progesterone (10, 100 and 1000 nM) for 4 days. Data are expressed as the means from three different experiments.

surface EGF receptors. The coincidence of the peak form in Caski cells and stromal cells in the presence of an EGF receptor antibody indicated that a specific binding component for the EGF peptide is present on the surface of intact endometrial stromal cells in culture.

To further characterize these surface EGF binding components of intact cells of the stromal monolayer competitive binding studies were performed. We have chosen a relatively low input of [$^{125}$I]EGF in our different experiments which might have negatively influenced the binding data. However, we were able to demonstrate in prestudy experiments that binding saturation under these experimental conditions was reached with an input of 60 000 c.p.m. [$^{125}$I]EGF or 0.19 nM [$^{125}$I]EGF respectively (data not shown). Therefore, all experiments were conducted with labelled EGF concentrations within this range. The increase of specific [$^{125}$I]EGF binding with time was furthermore an indicator for the existence of a surface binding component for the EGF peptide. The incubation of the monolayer with labelled [$^{125}$I]EGF in the presence of serial concentrations of unlabelled EGF, TGFrα and FGF further characterized the specificity of EGF binding sites, revealing the typical binding hierarchy of specific EGF receptors similar to binding studies in endometrial stromal cells and glands (Watson et al., 1994). TGFrα shows a 43.8% sequence homology with human EGF and displaces EGF in higher concentrations than EGF itself from the EGF receptor (Marquardt et al., 1983). In contrast the affinity of FGF to the EGF receptor is very low or even absent (Böhlen et al., 1984). Scatchard analysis of binding data indicated a dissociation constant ($k_D$) of ~0.36 nM. These data are in close agreement to the study by Watson et al. (1994) indicating a high affinity of EGF binding sites. However, this $k_D$ value is ~5-fold less than the value reported for isolated membranes (Singer and Strowitzki, 1996). This may be due to internalization and recycling of the EGF–receptor complex between intracellular organelles and the cellular surface. Internalization is inhibited in an isolated membrane preparation. Furthermore, the internalization process in intact monolayer cultures might explain the existence of a curvilinear plot resulting from competitive binding studies in intact monolayers. In membrane preparations a linear plot, indicating one class of high affinity binding sites, was obtained (Singer and Strowitzki, 1996). Taketani and Mizuno (1991) also described a linear plot of EGF binding in endometrial cells with a $k_D$ of 0.14 nM, comparable to the $k_D$ of high affinity binding sites in our study. However, no pure stromal
cell cultures were used in their study. Furthermore, we have used long-term endometrial stromal cell cultures passed at least four times in vitro. Therefore, different expression of low and high affinity binding sites in endometrial stromal cells after long-term incubation cannot be fully excluded. To further characterize the nature of the EGF binding component, we have performed an autoradiography after cross-linking iodinated EGF to its putative receptor in the intact monolayer. Our autoradiogram shows a radiolabelled protein band with an apparent molecular weight of ~170 kDa under reducing conditions, which has the same electrophoretic migration pattern as EGF receptor species already identified in A431 cells (Buhrow et al., 1982), endometrial stromal membranes (Singer and Strowitzki, 1996) and other tissues. Similar results were reported from freshly prepared membrane fractions of endometrial tissue samples (Reynolds et al., 1990). This similarity, as well as the formation of a single band under reducing conditions, confirms the molecular identity of these surface EGF-binding components as specific EGF receptor species.

In intact stromal cells, we have shown that incubation with progesterone modestly increases the binding of EGF. This might explain the necessity of EGF peptide for progesterone action in inducing the decidual transformation. Taketani and Mizuno (1991) also described a significant increase of [125I]EGF binding to a mixed culture of endometrial cells after a short-term incubation with progesterone and Reynolds et al. (1990) showed that progesterone might increase EGF binding in a pure culture of endometrial epithelial glands. These data are in accordance with a recent report from Imai et al. (1995) who demonstrated by reverse transcriptase–polymerase chain reaction in endometrial tissue samples that the expression of EGF receptors is significantly increased in late follicular and in luteal endometrium in comparison to the early follicular phase. Furthermore, in an immunohistochemical study maximum staining of stromal cells for EGF receptors was obtained in secretory endometrial samples (Wang et al., 1992). These data further emphasize the importance of EGF for endometrial proliferation in the follicular phase and for the secretory transformation. Recently, it has been shown that EGF binding increases in accordance with the decidualization process in vitro decidualized endometrial stromal cells (Taga et al., 1995).

However, an altered expression of EGF receptors in the phases of the menstrual cycle is controversial. Ace and Okulicz (1995) could not demonstrate a shift in the regulation of the EGF receptor gene in oestrogen- or progesterone-dominated endometria. Prentice et al. (1992) and Chegini et al. (1992) failed to demonstrate any cycle-dependent immunohistochemical staining of EGF receptors. In the rabbit Kleinlein et al. (1993) showed that an increase of endometrial EGF binding was dependent only on the exposure to oestrogens, whereas progesterone showed no significant effect.

In conclusion, intact endometrial stromal cells in monolayer culture possess typical EGF receptors, which are up-regulated by progesterone. These data provide further evidence that EGF is involved in the differentiation of secretory endometrium.

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References


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