Identification of mRNA for epidermal growth factor and transforming growth factor-α present in low copy number in human endometrium and decidua using reverse transcriptase-polymerase chain reaction


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RECEIVED 30 August 1990

ABSTRACT

The presence of mRNA for epidermal growth factor (EGF) and transforming growth factor-α (TGFα) was demonstrated in small fragments of human endometrium and decidua by use of the technique of reverse transcriptase-polymerase chain reaction with nested oligonucleotide primers. The presence of mRNA encoding EGF and TGFα has not been shown in human endometrium previously. Other studies using conventional techniques, such as Northern blot or in-situ hybridization, showed the presence in low copy number of EGF but not TGFα in murine endometrium. Messenger RNA for EGF was not present in peripheral leukocytes or platelets, suggesting an endometrial source for the message. Messenger RNA for TGFα was found in these blood components, thus preventing confirmation of the source of TGFα mRNA. Journal of Molecular Endocrinology (1991) 6, 207–214

INTRODUCTION

Proliferation of human endometrium is required to repair the denuded surface of the uterus after menstruation and to prepare the endometrium for the secretory changes needed for successful implantation. The paracrine or autocrine factors regulating this profound growth of uterine cells is poorly understood. Epidermal growth factor (EGF) is a potent stimulator of proliferation in many cell types, particularly fibroblasts, keratinocytes and epithelial cells (see Carpenter & Cohen, 1979; Burgess & Lloyd, 1988). Evidence that EGF may be involved in endometrial proliferation includes the observations that EGF receptors are present in rat (Mukku & Stancel, 1985; Lingham, Stancel & Loose-Mitchell, 1988) and human endometrium (Chegini, Rao, Wakim & Sanfilippo, 1986), prepro EGF mRNA and EGF immunoreactivity are present in mouse uterine epithelial cells (DiAugustine, Petrusz, Bell et al. 1988) and mouse uterine epithelial cell growth is enhanced by EGF (Tomooka, DiAugustine & McLachlan, 1986). Apart from the submaxillary gland (SMG) of the mouse and, to a lesser extent, the kidney (Rall, Scott, Bell et al. 1985), prepro mRNA is also present in low copy number in the uterus of the mouse (DiAugustine et al. 1988). Levels of EGF in humans are further reduced when compared with the mouse. In non-salivary glands, levels of immunoreactive EGF were 1% of the amounts found in the mouse, whilst in the SMG they were only 0.002% of the concentrations present in mice (Hirata & Orth, 1979). These authors did not demonstrate immunoreactive EGF in the uteri of women after cation exchange chromatography and radioimmunoassay. There are no cited reports of EGF or its mRNA being present in human endometrium.

In addition to EGF, transforming growth factor-α (TGFα) is a ligand for the EGF receptor, and significant levels of TGFα and TGFβ mRNA are found in self-renewing epithelial tissues, such as skin and gastrointestinal tract (Derynck, Roberts,
Winkler et al. 1984; Derynck, Goeddel, Ullrich et al. 1987; Malden, Novak & Burgess, 1989). However, although TGFα mRNA is expressed in stromal cells of rat decidua, it was not detected by either in-situ or Northern blot hybridization in non-pregnant rat endometrium (Han, Hunter, Pratt et al. 1987) and has not been previously demonstrated in human endometrium.

In addition to the already low levels of mRNA for EGF and TGFα present in uterine tissue, research into human disease is inevitably hampered by the small amounts of tissue that can be obtained at biopsy. However, the recently described technique of reverse transcriptase-polymerase chain reaction (RT-PCR) provides a very sensitive amplification method for demonstrating the presence of mRNA either from small volumes of tissue and/or where the mRNA is present in very low copy number. The aim of this study was to use RT-PCR to determine whether mRNA for EGF nad TGFα was present in small amounts of human endometrium.

MATERIALS AND METHODS

Materials

Sodium dodecyl sulphate (SDS), Tris buffer, 2-mercaptopethanol, EDTA, gelatin, proteinase K and sarkosyl were obtained from BDH (Poole, Dorset, U.K.). Guanidinium isothiocyanate was bought from Gibco BRL (Uxbridge, Middx, U.K.). Caesium chloride was purchased from Boehringer Corporation (Mannheim, Germany). Reverse transcriptase was bought from Anglian Biotech (Colchester, Essex, U.K.) and Taq polymerase from United States Biochemicals (USB; Cleveland, OH, U.S.A.). Neutralized deoxynucleoside triphosphates (dNTPs) were obtained from Pharmacia Ltd (Milton Keynes, Bucks, U.K.) and RNAsin (human placental ribonuclease inhibitor) was bought from Promega (Madison, WI, U.S.A.)

Preparation of total RNA from endometrium and decidua

Endometrium was obtained from uteri removed for benign conditions. Approval was granted by the ethical committee of the Cambridge District Health Authority. Term decidua was obtained from the uterine cavity at the time of Caesarian section. A sample of tissue was placed in formalin for histological identification. Total RNA was prepared from one sample of proliferative endometrium, two samples of early secretory endometrium and one sample of decidua.

Immediately on collection, tissue samples were quickly rinsed with ice-cold phosphate-buffered saline (PBS) to remove excess blood and flash-frozen in liquid nitrogen. Total RNA was isolated by Polytron homogenization of the tissue in 20 volumes guanidinium isothiocyanate (Chirgwin, Przybyla, Macdonald & Rutter, 1979). Following addition of solid caesium chloride (1 g/2.5 ml homogenate), the solution was carefully layered over a 2 ml density gradient 'cushion' of 5.7 M caesium chloride plus 100 mM EDTA (pH 7.0) in a Beckman quick-seal ultracentrifuge tube (Glisin, Crkvenjakov & Byus, 1974). After centrifugation at 110 000 g at 17 °C for 20 h, the total RNA pellet was resuspended in 10 mM Tris buffer plus 1 mM EDTA with 0.1% (w/v) SDS and incubated at 65 °C to aid dissolution. The RNA was precipitated by the addition of 0.1 volumes 3 M sodium acetate and 2.5 volumes absolute ethanol and placed on solid CO₂ for 10 min. Following pelleting by centrifugation, this procedure was repeated with final resuspension in sterile distilled water.

In order to investigate the role of peripheral blood cells in contributing mRNA for EGF or TGFα in the tissue, leukocytes from human peripheral blood were isolated using Lymphoprep (Nycomed, Sheldon, West Midlands, U.K.) by modification of the method of Boyum (1964). Briefly, 15 ml heparinized whole blood was layered over 10 ml Lymphoprep in a 50 ml Falcon tube. Care was taken not to disturb the interface when loading the blood from the Pasteur pipette. Samples were spun at 800 g for 15 min at 18–20 °C in a bench-top centrifuge with brake off, to maximize lymphocyte recovery. The sharp cream-coloured band of leukocytes was aspirated with a sterile Pasteur pipette and washed twice with 50 ml sterile PBS. The cell pellet was resuspended in 1 ml PBS and lysed by the addition of 10 volumes buffered guanidinium isothiocyanate (4 M guanidinium isothiocyanate, 50 mM Tris–HCl, pH 7.4, 0.2% (w/v) sarkosyl, 10 mM EDTA and 0.1% (v/v) 2- mercaptopethanol) followed by vigorous vortexing to disrupt the cells and shear the DNA as described by Chirgwin et al. (1979). The RNA was pelleted through 2 ml of a 5.7 M caesium chloride step gradient in a polycellor centrifuge tube. Centrifugation was performed in a 75Ti fixed-angle rotor for 17–20 h at 110 000 g at 17 °C. To minimize disturbance of the gradient, slow acceleration and deceleration was selected on the Beckman L8 ultracentrifuge.

To investigate the contribution of residual RNA present in platelets to the pool of RNA, a highly purified platelet preparation was established by filtering a leukocyte preparation through a 0.45 μm Sepacell filter (Kimmel Scientific Products Ltd, Uxbridge, Middx, U.K.) to remove the leukocytes.
Total RNA was extracted by suspending 0·1 ml of the platelet concentrate in 0·1 ml digestion buffer (10 mm Tris–HCl, pH 8·0, 10 mm EDTA, 50 mm NaCl and 2% SDS). Proteinase K (15 μl; 10 mg/ml) was added to the mixture (10 mg/ml water) which was incubated at 56 °C for 3 h in a 2 ml Eppendorf tube. The digest was repeatedly extracted with equal volumes of phenol and chloroform:isoamylalcohol (24:1) until the interface was clear. This required seven extractions involving brief vortexing, followed by spinning in a microcentrifuge for 2 min and transferring the top (aqueous) layer to a new Eppendorf tube. Residual phenol/chloroform was removed by extracting with an equal volume of water-saturated n-butanol. The lower aqueous layer was concentrated by ultracentrifugation in a Centri- con 30 device (Amicon, Gloucester, Glos, U.K.) to a volume of 35 μl.

**cDNA synthesis**

First-strand cDNA was synthesized without prior poly(A) + mRNA purification. Between 2 and 10 μg total RNA were annealed to oligo(dT) primer (100 μg/ml final concentration) by heating to 68 °C for 5 min, then chilling on ice for 2 min. After addition of dNTPs (final concentration 1 mm), 1 × reverse transcriptase buffer (50 mm Tris–HCl, pH 8·3 at 42 °C, 40 mm KCl, 6 mm MgCl₂ and 4 mm dithiothreitol), 1 μl (20 units) RNAsin and 0·5 μl (10 units) avian myoblastosis virus reverse transcriptase enzyme in a final volume of 20 μl were added. First-strand cDNA synthesis was carried out at 42 °C for 1 h (Gubler & Hoffman, 1983). The reverse transcriptase was inactivated by heating at 80 °C for 10 min before using the cDNA.

**Polymerase chain reaction (PCR)**

One-tenth of the cDNA (2 μl) was subject to PCR amplification using a Techne PHC-1 programmable thermocycler (Techne (Cambridge) Ltd, Duxford, Cambs, U.K.). Oligonucleotide primers were synthesized on an Applied Biosystems 380B DNA synthesizer (Applied Biosystems, Foster City, CA, U.S.A.) and purified by ethanol precipitation.

The amplification profile for 35 cycles was dissociation at 95 °C for 0·3 min, annealing at 55 or 62 °C for 0·5 min and extension at 72 °C for 0·5 min. The final cycle included polymerization for 5 min for complete strand extension. A 50 μl reaction mix, overlaid with 50 μl light mineral oil, contained 2 μl cDNA, 5 μl 10 × reaction buffer (100 mM Tris–HCl, pH 8·3 at 25 °C, 500 mM KCl, 15 mM MgCl₂ and 0·1% gelatin), 5 μl 10 × dNTPs (2 mm), 5 μl each oligonucleotide primer (1·0 μm final concentration), 2·5 units Taq polymerase and sterile double-distilled water to 50 μl. The total reaction mix was heated to 95 °C for 2 min before adding the Taq polymerase to inactivate any contaminating nucleases and protases.

Nested PCR was performed by taking 1 μl reaction mixture from the first round of amplification and subjecting it to a further 20 cycles of amplification in a fresh 50 μl reaction using the internal primer pair.

**Oligonucleotide design**

The two pairs of forward and reverse primers synthesized to anneal with cDNA for EGF and TGFα are shown in Table 1. External primers were designed to span a known intron, such that amplification of the predicted size minus the intron could be taken as representative of tissue expression. The intron/exon boundary in the EGF cDNA lies between bases 3383 and 3384 and for TGFα between bases 127 and 128. The experimental design of the nested PCR reaction is shown in Figs 1a and 2a, where the primers A, B, C and O are as shown in Table 1.

**DNA cloning**

A 5 μl aliquot of the PCR products was subjected to agarose gel electrophoresis to determine the number

**TABLE 1. Oligonucleotide sequences of the external and internal, forward and reverse primers used to perform nested polymerase chain reaction to identify cDNA for epidermal growth factor (EGF) and transforming growth factor-α (TGFα), indicating the complementary positions of the published sequences for EGF and TGFα**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Base</th>
<th>Nucleotide sequence</th>
<th>Base</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) EGF forward</td>
<td>~3261</td>
<td>GACGCCTGTCTGAACAGGA</td>
<td>~3281</td>
</tr>
<tr>
<td>(B) EGF reverse</td>
<td>~3628</td>
<td>CGATAGCAAGCTCTGAGTCC</td>
<td>~3606</td>
</tr>
<tr>
<td>(C) EGF forward</td>
<td>~3321</td>
<td>AAGATGACCACTATTTCCG</td>
<td>~3341</td>
</tr>
<tr>
<td>(D) EGF reverse</td>
<td>~3573</td>
<td>AGCATGACAAAGCACCACACCAC</td>
<td>~3554</td>
</tr>
<tr>
<td>(A) TGFα forward</td>
<td>~63</td>
<td>TGCTTCCTGCTGCTATTTGGTG</td>
<td>~82</td>
</tr>
<tr>
<td>(B) TGFα reverse</td>
<td>~383</td>
<td>GTATGATAAGGACAGGCCAGG</td>
<td>~364</td>
</tr>
<tr>
<td>(C) TGFα forward</td>
<td>~113</td>
<td>ACGTCCCGCTGAGTGCAGA</td>
<td>~132</td>
</tr>
<tr>
<td>(D) TGFα reverse</td>
<td>~349</td>
<td>CACCAAGGGGCGGTGATGG</td>
<td>~330</td>
</tr>
</tbody>
</table>

The letters A, B, C and D refer to the same oligonucleotides shown in Figs 1a and 2a.
of bands and their size and yield, before phenol-
chloroform extraction and ethanol precipitation of
the remaining product. To permit efficient cloning,
DNA was end repaired with T4 DNA polymerase to
ensure blunt ends. This process is necessary because
DNA polymerases including that isolated from
*Thermus aquaticus* can add, at a low rate, nucleotides
to the 3-OH group at the end of a double-stranded
DNA molecule (Clark, 1988). The products were
then ligated into prepared Bluescript KSk+ plasmid
vector (Stratagene, La Jolla, CA, U.S.A.) and
transformed into frozen competent *Escherichia coli*
NM522 or TG1 cells (Hanahan, 1983). The resulting
colonies were screened for the presence of the
correct inserts by PCR (Schofield, Vaudin, Kettle &
Jones, 1989), and mini-preparation plasmid DNA
was prepared as described by Jones & Schofield

**Mini-preparation of plasmid DNA**

A modified alkaline-lysis procedure (Birnboim &
Doly, 1979) was developed to facilitate the prepara-
tion of DNA for sequencing (Jones & Schofield,
1990). The main advantages of this approach are
that there is no requirement for the addition of
lysozyme, no organic extraction steps and high-
quality DNA is prepared for dideoxy sequencing.
From an overnight 2 ml culture, the yield of DNA is
4–5 μg which is sufficient for a minimum of five
sequencing reactions. The culture was transferred to
a 2.2 ml Eppendorf tube and centrifuged for 1 min at
8000 *g*. The bacterial pellet was resuspended in
200 μl of a mixture of 50 mM glucose, 25 mM Tris-
HCl (pH 7.4) and 10 mM EDTA, and 400 μl alkali
SDS were added (0.2 M NaOH plus 1% SDS). This
was incubated at room temperature for 5 min, after
which 300 μl 3 M potassium acetate (pH 4.8) were
added. After incubation on ice for 5 min this was
centrifuged at 8000 *g* for 5 min and the supernatant
transferred to a clean 2.2 ml tube which was then
filled with 1 ml ethanol. This was mixed and
immediately centrifuged for 5 min. The pellet was
washed once with 70% ethanol, dried and resus-
pended in 40 μl sterile water. A portion of this DNA
(8 μl) was used for sequencing as described below.

**DNA sequencing**

Double-stranded plasmid DNA sequencing was
performed by the dideoxy chain-termination
method (Sanger, Nicklen & Coulson, 1977) using a
modification of the Sequenase (USB) protocol in
which the DNA is denatured at 37 °C for 10 min in
0.2 M NaOH, and the primer was annealed to the
denatured template before precipitation (Chi, Hsueh, Hui & Tam, 1988). Following development
of the autoradiogram, the DNA sequence was
computer aligned with the previously published
DNA sequences for EGF and TGFα, using the
DNAid program (Dardel & Bensoussan, 1988).

**RESULTS**

The most significant result of the PCR was that
only by performing a second nested PCR on the cDNA
was it possible reliably to obtain a dominant species
of the predicted length (Figs 1b and 2b). Prior to
this, optimization of the reaction conditions was
attempted by adjustment of cycle annealing tem-
peratures, cycle number, magnesium concentrations
and synthesis of other primer pairs. Despite all such
attempts, the primary PCR, using the external
primers only, resulted in multiple non-specific
bands (Figs 1b and 2b, lanes 1 and 3). Nested PCR
gave reproducible dominant target products of the
predicted sizes for EGF and TGFα (Figs 1b and 2b,
lanes 2 and 4) which were readily cloned after end
repair with T4 DNA polymerase.

PCR of cDNA prepared from circulating leuko-
cytes and platelets was negative for EGF (results
not shown) but positive for TGFα (Fig. 3). PCR was
undertaken without input cDNA to exclude the
possibility of contamination of the primers which
is a significant problem during PCR (Sarkar &
Sommer, 1990).

Mini-preparation of plasmid DNA was achieved
by a modification of the alkaline-lysis method,
allowing a more rapid procedure whose DNA
provided an excellent sequencing template. The
sequence of the TGFα PCR product showed
complete identity with the published sequence. The
sequenced EGF PCR product had a single silent
base change which could represent either a polymor-
phism or a PCR artifact (Figs 1c and d and 2c and d).

**DISCUSSION**

This study demonstrates that human endometrium
contains cells that express mRNA for both EGF and
TGFα. In the case of EGF, this extends the
observations in the murine uterus (DiAugustine
*et al.* 1988) to that of the human. The inability to
derive a PCR product from leukocytes and platelets
suggests that the source of the mRNA for EGF is
either from specific endometrial cells or cells derived
from another source which are transformed when
they enter the endometrium. It is not possible from
this study to determine whether the glandular or
stromal cells are the source of the mRNA for EGF.
However, this study does suggest that endometrium
is a source of EGF and, in view of the previous demonstration of receptors for EGF on both glandular and stromal cells of human endometrium (Chegini et al. 1986), it is possible that EGF of uterine origin may have a paracrine role in human endometrial proliferation.

Hirata & Orth (1979) failed to show the presence of immunoreactive EGF in human endometrium, but these findings suggest that their technique was not sensitive enough to demonstrate the low levels of peptide probably present in normal human endometrium. Previous studies in mammalian species have shown that the mRNA for prepro EGF is present in the uterus in low copy number (DiAugustine et al. 1988). RT-PCR provides a new and sensitive technique for investigating the expression of very low levels of mRNA encoding EGF in small volumes of tissue.

Expression of TGFα was initially described in retrovirus-transformed fibroblasts (Todaro, Fryling & DeLarco, 1980) and later in squamous carcinomas (Derynck et al. 1987). Recent studies, however, have shown that it is synthesized by normal epithelial skin keratinocytes (Coffey, Derynck, Wilcox et al. 1987).
RT-PCR has been used to demonstrate mRNA for TGFα in both mouse fetal tissue and activated wound macrophages (Rappolee, Brenner, Schultz et al. 1988a; Rappolee, Mark, Banda & Werb, 1988b). In rats, Northern blot hybridization failed to demonstrate the presence of mRNA for TGFα in non-pregnant uteri (Han et al. 1987) probably because of the very low levels of message. The identification of mRNA for TGFα not only in endometrium and decidua but also in peripheral leukocytes and platelets prevents identification of the source of the message as being from the glandular or stromal compartments of the endometrium. However, endometrial stroma contains both macrophages and large granular lymphocytes (Bulmer, Hollings & Ritson, 1987; Kamat & Isaacson, 1987) which infiltrate the stroma in the secretory phase of the cycle and are particularly abundant in the premenstrual phase of the cycle (Bulmer, Lunny & Hagin, 1988). In rat decidua, in-situ hybridization demonstrated the peptide in stromal but not epithelial cells (Han et al. 1987).

This study demonstrates the power of RT-PCR to identify the presence of mRNA for peptides when the message is present in low copy number. Unlike Northern blot hybridization and dot blots, it does not require large amounts of tissue, as there is no need for the preparation of poly(A)+ RNA. The technique of nested internal PCR further enhances the resolution of DNA products generated by the PCR and improves the reliability of the technique. In addition, investigation of the presence of very low levels of mRNA in tissue specimens by RT-PCR should include examination of amplified cDNA from blood products.

Inherent in a technique as sensitive as RT-PCR is
the problem of false positive findings due to contaminating template. In this study, cDNA contamination was unlikely as cloned human EGF or TGFα cDNA has never been introduced to the laboratory, amplification of genomic DNA was prevented as primers were designed to span known intron/exon boundaries and PCR using the primers in the absence of template DNA failed to show a product.

Considering the biological efficacy of growth factors at very low concentrations and the small amounts of peptide that are probably required to exert paracrine or even autocrine effects, this study demonstrates that great caution must be employed when ascribing physiological significance to the absence of mRNA for a peptide if Northern blot or in-situ hybridization studies are employed. However, because of the difficulty of quantifying the original tissue levels of mRNA after gene amplification, great caution must also be used when attributing physiological significance to these observations.

Epidermal growth factor may be involved in the process of epithelial regeneration that occurs at menstruation but, in addition, human placenta expresses the EGF receptor (Lai & Guyda, 1984) and EGF could induce trophoblastic proliferation. As most of the surface epithelium and two thirds of the glandular epithelial cells are lost at menstruation (Nogales-Ortiz, Puerta & Nogales, 1978) it is also possible that TGFα, expressed by activated macrophages or platelets present on the denuded surface of the endometrium, is in part responsible for the epithelial regeneration that is complete by day 5 of the menstrual cycle (Ludwig & Metzger, 1976).

These findings demonstrate for the first time that human endometrium and decidua contain cells which contain mRNA for EGF and TGFα. Further studies are now required to quantify PCR, in order to study discrete changes of mRNA levels in small fragments of human tissue which may be obtained without the need for operative procedures.

**ACKNOWLEDGEMENTS**

We should like to thank the consultants and staff of Addenbrooke’s Hospital, Cambridge for their cooperation in this study. R.B.H. was supported by a grant from Hoechst Pharmaceuticals and J.R.-W. by a grant (S1/89) from Birthright. J.P.S. was supported by an MRC Training Fellowship.

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