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Kappa opioids and TGFβ1 interact in human endometrial cells

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The transforming growth factor β1 (TGFβ1) is a major regulator of human endometrial function. Human endometrium possesses specific opioid binding sites, the majority of which belong to the kappa type, for which the prodynorphin-derived opioids are the endogenous ligands. Since these two systems interact in several other tissues we postulated that opioids may affect the production of TGFβ1 in human endometrium. We have found that kappa opioids exerted a time- and dose-dependent inhibitory effect on TGFβ1 production from endometrial stromal and epithelial cells and from the Ishikawa human endometrial adenocarcinoma cell line. This effect was reversible by the specific opioid antagonist diprenorphine. To examine if this effect represents a paracrine endometrial response to locally produced kappa opioids we searched for the presence of the endogenous kappa opioid receptor ligands. Indeed, the prodynorphin transcript was detectable on Northern blots from normal and tumoral human endometrial cells; its size was that of the pituitary transcript, i.e. ~2.4 kb long. Most immunoreactive dynorphin from human endometrium had a molecular weight of 8 kDa. Finally, immunofluorescence staining of normal and tumoral human endometrial cells revealed the presence of dynorphin-positive cytoplasmic secretory granules. Taken together, our data suggest that in human endometrium, kappa opioids and the TGFβ1 form a paracrine network which appears to be retained by the Ishikawa human endometrial adenocarcinoma cell line.

Key words: dynorphins/endometrium/human/opioids/TGFβ

Introduction

Multiple lines of evidence suggest that the transforming growth factor (TGF) is an important modulator of endometrial physiology involved in the regulation of endometrial growth, proliferation, and differentiation (Godkin and Dore, 1998). Indeed, endometrial TGFβ1 affects the proliferation of normal human epithelial and stromal endometrial cells as well as the proliferation of the human endometrial adenocarcinoma cell line Ishikawa (Croxtall *et al*., 1992; Tang *et al*., 1994; Albright and Kaufman, 1995). Following ovulation, TGFβ1 stimulates stromal cell proliferation, while at the same time inhibiting endometrial epithelial cellular mitosis promoting their differentiation to the characteristic mature cells of the secretory endometrium (Marshburn *et al*., 1994). In addition, TGFβ1 promotes apoptosis of endometrial stromal cells at the implantation site in an effort to accommodate the developing trophoblast in a hospitable implantation chamber (Moulton, 1994).

Human endometrium produces opioids and their receptors suggesting that endometrial opioids exert local, autocrine/paracrine, effects. Indeed, transcripts of endogenous opioid precursors proopiomelanocortin (POMC) and proenkephalin (PENK) and their end products have been reported in human endometrial cells (Walhstrom *et al*., 1985, Petraglia *et al*., 1986, Makrigiannakis *et al*., 1992). Multiple types of opioid binding sites have been identified in human endometrial cells, with the kappa (κ)-opioid receptor (κ1, κ2, κ3) being the predominant type, while κ-opioid receptors are present in lower numbers and the κ-type of receptors are not detectable (Hatzoglou *et al*., 1995a).

Since these two systems interact in several other tissues we postulated that opioids may affect the production of TGFβ1 in human endometrium. Indeed in rat brain, κ-opioids are co-localized and interact with TGFα and β (Code *et al*., 1987; Ramirez-Ordonez *et al*., 1999). Furthermore, κ-opioids stimulate the release of TGFβ from human peripheral blood mononuclear and porcine immune cells (Chao *et al*., 1992; Zhou *et al*., 1992). The effects of opioids on TGF are not unique but they are rather part of a more generalized effect of opioids on growth factors. Thus, it has been shown that synthetic κ-opioid agonists can interact with nerve growth factor (NGF) in the rat pheochromocytoma cells (Margioris *et al*., 1992); inhibit macrophage-colony stimulating factor (M-CSF) in mouse bone marrow cells (Roy *et al*., 1991); and modulate the biological effects of epidermal growth factor (EGF) in monkey kidney cells (Belcheva *et al*., 1998).

In the first part of this study, we examined the effect of synthetic opioid agonists and antagonists on the production of TGFβ1 by normal human endometrial cells and the Ishikawa human endometrial adenocarcinoma cell line. In the second part (following our data showing a specific effect of κ-opioid agonists on endometrial TGF), we examined whether the endogenous ligands for the κ-opioid receptors are produced in

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normal and tumoral human endometrium. We have found that human endometrium produces dynorphins. Taken together our data suggest that κ-opioids and TGF interact in a paracrine mode at the level of human endometrium.

Materials and methods

Primary culture of isolated epithelial and stromal cells from human endometrium

Endometrial specimens were obtained from patients undergoing diagnostic curettage or hysterectomy. Full ethics committee approval had been granted for this study. The histological type and pathology of the biopsies was confirmed by conventional pathological examination. Purified epithelial and stromal primary endometrial cell cultures were established as described previously (Chatzaki *et al*., 1994; Makrigiannakis *et al*., 1995). Briefly, the tissues were collected in Dulbecco's minimal essential medium (DMEM; Gibco BRL, Bethesda, MD, USA) supplemented with 10% fetal calf serum (FCS) and 1% antibiotic/antimycotic solution, trimmed and minced mechanically and digested for 90 min at 37°C using 0.25% of Type I collagenase (Sigma Chemical Co, St Louis, MO, USA). Separation of the epithelial and stromal fraction was carried out by filtration through a 45 μ stainless steel sieve and backwashing of the glands from the sieve, followed by pelleting by centrifugation. The two different cell types were then plated in 24-well plates (Corning Inc, New York, NY, USA) with 1 ml of culture medium and incubated in a humidified atmosphere of 5% CO_2 at 37°C, in the above mentioned medium for 2 days before experimentation. At the beginning of the experiment, cultures had reached 100% confluency and at least 90% purity in epithelial or stromal cell content, as judged by light microscopy. Following this, they were pre-incubated in Phenol Red- and serum-free Roswell Park Memorial Institute (RPMI) 1640 medium (Biochrom Co, Berlin, Germany) supplemented with 0.25% bovine serum albumin (BSA) fraction V (Sigma), insulin from bovine pancreas (5 mg/l), transferin (5 mg/l) and sodium selenite (5 ng/l) (ITS cell culture supplement, Sigma), 2 mmol/l L-glutamine and 1% antibiotic/antimycotic solution for 24 h. The κ 1-opioid receptor agonist U69593 (Upjohn Co, Kalamazoo, MI, USA) and/or the general opiate antagonist diprenorphine (Sigma) were then added in the above medium in duplicate or triplicate wells, in parallel with non-treated controls.

Culture of the Ishikawa endometrial cell line

Ishikawa cells were established as a permanent cell line from a well differentiated human endometrial adenocarcinoma (Nishida *et al*., 1979; Gravanis and Gurpide, 1986). The cells were routinely cultured in 75 ml cell culture flasks (Corning) in Earle's minimal essential medium (Gibco) supplemented with 15% fetal bovine serum (FBS; Gibco), 10 mmol/l L-glutamine and 1% antibiotic–antimycotic solution (Gibco) to a final concentration of 100 IU/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 5%CO_2 at 37°C. Cells growing exponentially were seeded into 24-well plates (Corning) (25×10^3 cells/well) and incubated for 24 h in 1 ml growth medium. Before drug treatment, cells were placed in serum-free, Phenol Red-free medium as mentioned above.

Measurement of TGFβ1

At the end of the treatment period, cell culture supernatants were collected and stored at –70°C for TGFβ1 measurement, whereas cells were harvested in a standard trypsin solution (Gibco) and stored at –20°C for estimation of protein content. TGFβ1 was measured using a human TGFβ1 Quantikine immunoassay kit (R&D Systems, Oxon, UK), following the manufacturer's protocol. This includes an acidification step that activates latent TGFβ1 to immunoreactive TGFβ1 and a standard quantitative sandwich enzyme immunoassay technique. The sensitivity of the assay is 7 pg/ml. Results were expressed as pg/ml per μg of total cellular protein, determined on whole cellular homogenates by the Bradford method (Bradford, 1976), using BSA as standard.

Northern blot analysis

Total RNA was extracted from frozen tissues or cultured cells by the guanidine thiocyanate method (Maniatis *et al*., 1989). Following size-fractionation of RNA (50 μg/lane) by electrophoresis through 1.5% agarose gels containing 6% formaldehyde and 2 μg/ml ethidium bromide, gels were viewed under UV light to assess the integrity. After transfer to GeneScreen nylon filters (New England Nuclear, Boston, MA, USA), they were prehybridized and hybridized as previously described (Maniatis *et al*., 1989). A synthetic 48-mer oligonucleotide against bases 36–83 of the rat prodynorphin (*PDYN*) mRNA (this area of mRNA is identical in both humans and rats) (Douglass *et al*., 1989) was used as a probe that was labelled at the 3′ end with [γ-^{32}P]-deoxyadenocine triphosphate (800 Ci/mmole; Amersham, Arlington, IL, USA) and terminal deoxynucleotidyl transferase (BRL, Bethesda, MD, USA) to a specific activity of ~10^8 dpm/mg. Blots were washed in 0.2× SSC, 0.1% SDS for 30 min at 60°C. Autoradiography using Kodak XR film took place at –70°C in the presence of an intensifying screen. The approximate size of mRNAs was determined relatively to 18S and 28S rRNAs.

Gel filtration chromatography and radioimmunoassay for dynorphins

Peptides in Ishikawa cell culture medium and endometrial homogenates were concentrated by a C-18 reverse phase column (Sep-Pak; Waters Associates, Milford, MA, USA). Briefly, culture media and cellular homogenates were acidified by 10 volumes of 0.1 N HCI and centrifuged at 5000 *g* for 10 min. The supernatants were extracted by activated Sep-Pak cartridges, washed with 20 ml 0.1 N HCl, eluted with 3 ml acetonitrile 80%–0.01% HCI, then dried under vacuum (Speed-Vac). The recovery of synthetic dynorphin diluted in medium using this method was >90%. Samples were then reconstituted in 0.5 ml 10% formic acid containing 0.5% defatted BSA and 6 mol/l urea and chromatographed on a Sephadex G-50 column (0.9×60 cm, bed volume 40 ml). The G-50 column was calibrated with blue dextran and dynorphin-$_{(1–13)}$. With flow rate 1.5 ml/h, fractions of 1 ml were collected, dried under vacuum and reconstituted for radioimmunoassay using an antiserum raised against synthetic porcine dynorphin-$_{(1–13)}$, which is identical to human dynorphin-$_{(1–13)}$ (Suda *et al*., 1983; Margioris *et al*., 1992). The antiserum cross-reacts with human dynorphin-$_{(1–13)}$ and dynorphin A. It exhibits no cross-reaction with synthetic human β-endorphin, neo-endorphin, or met- or leu-enkephalin. The sensitivity of the assay was 1 pg/tube.

Indirect immunofluorescence

Immunofluorescence staining was performed as described previously (Fostinis *et al*., 1992). Briefly, cells grown on 22×22 mm coverslips in absence of serum were fixed with acetone/methanol (9:1) for 20 min. The cells were then incubated for 1 h at 4°C with rabbit antiserum against human dynorphin, diluted 1:250 in phosphate-buffered saline (PBS) 0.1% BSA (Margioris *et al*., 1992) in parallel with a negative control (PBS 0.1% BSA). After washing, an anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC) was added (1:150, Amersham) for 1 h at 4°C. Specimens were visualized in a Zeiss Axioscope microscope and photographed using TMZ 135–36 Kodak film.

Statistical analysis

Data of TGFβ1 concentrations are presented either as pg/ml per µg of protein or as percentage of non-treated controls. For the statistical analysis of the results we used analysis of variance followed by post-hoc comparison of means and least significant difference test or planned comparison. All analyses were carried out on combined results from at least three identical experiments, performed in duplicate or triplicate.

Results

Effects of opioid agonists and antagonists on TGFβ1 production from primary cultures of human endometrial cells

Purified primary cultures were established after isolation of the respective cellular fraction from endometrial biopsies, obtained from patients undergoing diagnostic curettage. As we have previously reported, our method provides a pure population of isolated epithelial or stromal monolayers, with little contamination from other uterine cell types (Makrigiannakis *et al.*, 1995). We investigated TGFβ1 production from epithelial and stromal cell primary cultures of human endometrium together with the effect of κ-opioid peptides on endometrial TGFβ1. For this purpose, the use of synthetic opioid receptor ligands was preferred to dynorphin peptides, due to their stability *in vitro*. U69593 is a non-peptidic synthetic molecule that exhibits specific agonist activity on κ_1-opioid receptors and was therefore suitable for the purpose of these experiments. The concentration of TGFβ1 was measured in the culture supernatant after 2 days of treatment with U69593 (10^{-7} mol/l) in parallel with non-treated controls and values were corrected for total protein content.

The concentration of TGFβ1 in media under basal conditions or after opioid agonist application is presented in Figure 1. TGFβ1 was secreted from all tissues studied and from both epithelial and stromal cell fraction. The mean values of the basal concentration of TGFβ1 from each cell type cultured for 2 days were expressed as pg/µg of protein ± SEM, and were 14.97 ± 5.27 for proliferative epithelium (PE, n = 7), 10.62 ± 2.66 for secretory epithelium (SE, n = 5), 146.73 ± 52.21 for proliferative stroma (PS, n = 4) and 61.26 ± 14.15 for secretory stroma, (SS, n = 5) (Figure 1A). It is noted that the stromal fraction secreted higher levels than the glandular in both phases of the cycle. This finding corroborates with other reports showing that stroma is the principal source of TGFβ1 in the uterine cavity (Chegini *et al.*, 1994; Marshburn *et al.*, 1994).

Effect of opioid agonists and antagonists on TGFβ1 production from human endometrium cells in culture

Incubation of various endometrial cell types with 10^{-7} mol/l U69593 caused a significant reduction in the concentration of TGFβ1 in the culture supernatant of almost all primary cultures (Figure 1B). Indeed, this effect was observed in seven out of seven of PE, three out of five SE, four out of four PS and four out of five SS. As shown in Figure 1, the inhibitory effect of the opioid agonist was at much the same level (52 ± 9 to 69 ± 5% of non-treated controls) in all types of cells. We further explored the observed effect in time- and dose-response experiments, using cells from both phases of the cycle (Figure 2). Time–response curves (Figure 2A) showed that the statistically significant reduction of TGF-β1 secretion by U69593 was abolished after 6 days of culture of epithelial cells and 4 days of culture for stromal cells. Thus, 2-day treatment was chosen for all the following experiments. Also, it was noticed that basal release of TGFβ1 decreased during the 6 days of culture, probably reflecting a decrease in the number of viable cells, due to prolonged incubation in serum-free medium with no oestrogen supply that cannot support endometrial growth for long. However, the use of this medium was necessary, because serum contains measurable levels of TGFβ1, as well as other growth factors and steroid residues that could affect the regulation of TGFβ1 expression (Gutierrez *et al.*, 1998). Dose–response curves were biphasic for both stromal and epithelial cells (Figure 2B). An inhibitory effect of U69593 was observed at 10^{-10} mol/l (0.1 nmol/l) and at 10^{-8} and 10^{-7} mol/l (10 and 100 nmol/l), but not at 10^{-9} mol/l (1 nmol/l). When further dilutions of the drug were tested, it was shown that the

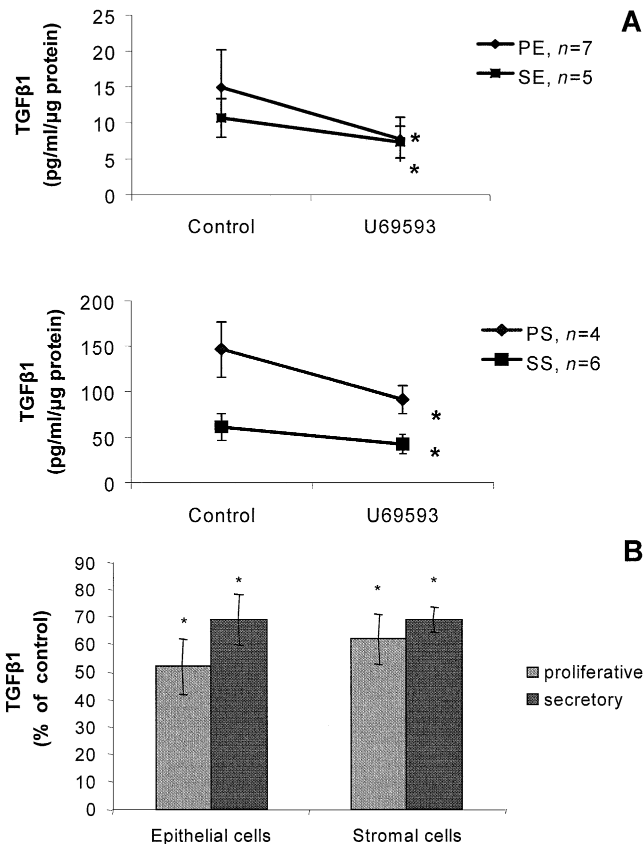

Figure 1. Effect of the synthetic κ-opioid receptor agonist U69593 on the secretion of transforming growth factor (TGF)β1 by epithelial and stromal cells of normal human endometrium. (**A**) The absolute values of the secreted TGFβ1 in the culture media in basal conditions and after 2 days exposure to 10^{-7} mol/l U69593. (**B**) The same results expressed as a percentage of non-treated controls. The bars represent the mean values expressed as percentage of non-treated controls and the error bars show the standard errors between cultures. PE = proliferative epithelial cells; SE = secretory epithelial cells; PS = proliferative stromal cells; SS = secretory stromal cells. *Statistically significant differences from the non-treated control ($P < 0.05$).

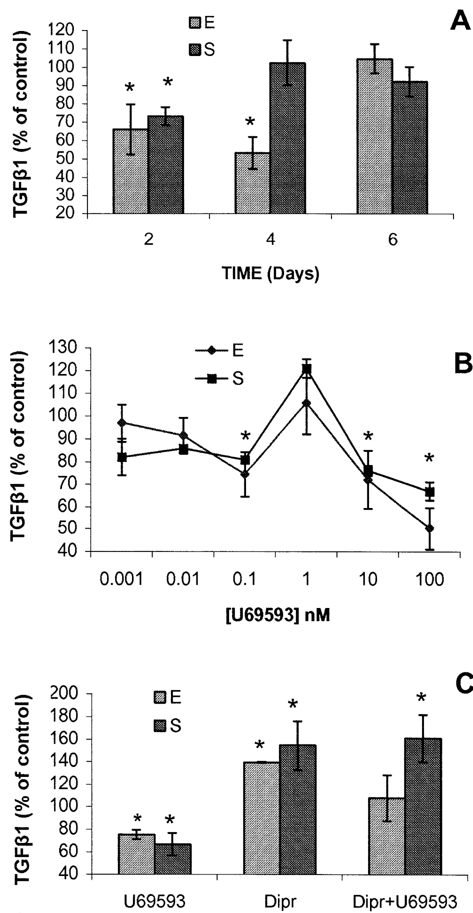

Figure 2. Effect of the synthetic κ-opioid receptor agonist U69593 on the secretion of transforming growth factor (TGF)β1 by epithelial and stromal cells of normal endometrium. Cells were treated with U69593 93 (**A** and **C** 10^{-7} mol/l and **B**), in presence or absence of the opioid antagonist diprenorphine (Dipr, 10^{-6} mol/l) (**C**) in parallel with non-treated controls and the concentration of TGFβ1 was measured in the culture media after 2, 4 and 6 days of treatment (**A**), or just on the second day (**B** and **C**). The bars represent the mean values of three experiments from different endometria, expressed as a percentage of non-treated controls and the error bars show the standard errors between replicates. E = epithelial cells; S = stromal cells. *Statistically significant differences ($P < 0.05$).

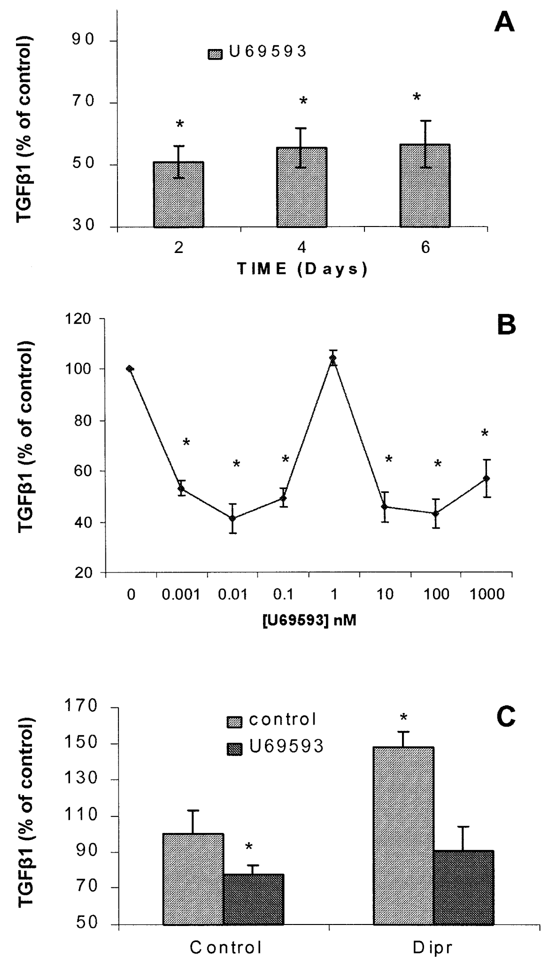

Figure 3. Effect of the synthetic κ-opioid receptor agonist U69593 on the secretion of transforming growth factor (TGF)β1 by Ishikawa human endometrial cells. Cells were treated with U69593 (**A** and **C** 10^{-7} mol/l and **B**), in the absence or presence of the general opioid antagonist diprenorphine (Dipr, 10^{-6} mol/l) (**C**) in parallel with non-treated controls and the concentration of TGFβ1 was measured in the culture medium after 2, 4 and 6 days of treatment (**A**), or just on the second day (**B** and **C**). The bars represent the mean values of three identical experiments expressed as a percentage of non-treated controls and the error bars show the standard errors between replicates. *Statistically significant differences ($P < 0.05$).

inhibitory effect was abolished in the nanomolar concentrations of U69593 ranging from 2×10^{-10} to 10^{-9} mol/l, whereas in 2×10^{-9} mol/l the effect was statistically significant (data not shown). As discussed later, this interesting profile probably reflects interaction of the ligand with multiple types of opioid or other receptors. In order to test whether the observed effect is mediated through specific opioid receptors, we measured TGFβ1 release after 2 days of treatment of various endometrial cell types with U69593 (10^{-7} mol/l) in the presence or absence of a ten-fold excess of the opioid antagonist diprenorphine (10^{-6} mol/l). Diprenorphine was chosen due to its general opioid receptor antagonistic activity compared with the more often used naloxone, a μ-preferential antagonist (Billington *et al.*, 1990). Naloxone has also been reported to exhibit also agonist activity (Cruz *et al.*, 1996). As shown in Figure 2C, the inhibition caused by U69593 was reversed in the presence of the antagonist, diprenorphine, suggesting action through opioid receptors. It is interesting to note that treatment with diprenorphine alone increased TGFβ1 secretion in a statistically significant way, suggesting that the action of exogenously produced opiods is blocked.

Effect of opioid agonists and antagonists on TGFβ1 production from Ishikawa human endometrial adenocarcinoma cells in culture

Ishikawa cells were treated with U69593 (10^{-7} mol/l) and the concentration of TGFβ1 was measured in the culture media after 2, 4 and 6 days of treatment. The combined results of three identical experiments are illustrated in Figure 3A. A statistically significant reduction of the concentration of TGFβ1 was observed, with a maximum of 52.02 ± 5.26% of non-treated control ($P < 0.05$) on the second day of treatment. The effect remained similarly profound after 4 and 6 days of

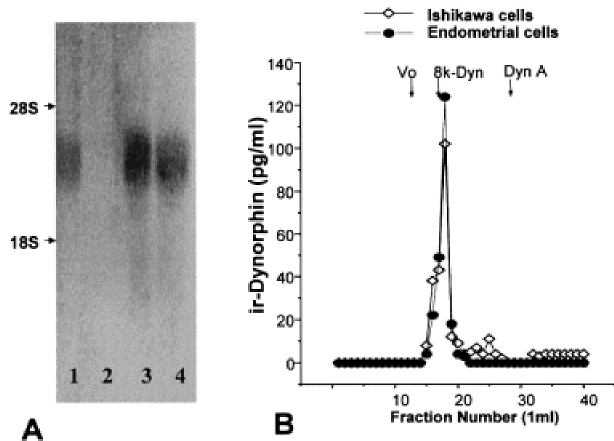

Figure 4. (**A**) Northern blot analysis of total RNA for the detection of prodynorphin transcripts: rat pituitary (lane 1), rat liver (lane 2), Ishikawa human endometrial adenocarcinoma cells (lane 3) and normal human endometrial tissue (lane 4). (**B**) Sephadex G-50 gel filtration chromatography of human endometrial cell homogenate and culture media conditioned by Ishikawa cells. The arrows indicate the void volume (Vo) and the elution positions of human 8 kDa and 2 kDa dynorphins (8 kDa Dyn and Dyn A respectively).

treatment (55.57 ± 6.30 and 56.64 ± 7.53 respectively, $P < 0.05$). The dose–response curve of this effect, measured after 2 days of treatment, is presented in Figure 3B. The profile of the curve was biphasic, confirming the results from the primary cultures of endometrial cells. Indeed, although we were able to detect a significant decrease in concentrations as low as 10^{-12} mol/l (0.001 nmol/l), at the concentration of 10^{-9} mol/l (1 nmol/l), the effect was abolished. Finally, we measured TGFβ1 release after 2 days of treatment with U69593 (10^{-7} mol/l) in the presence or absence of a 10-fold excess of the opioid antagonist diprenorphine (10^{-6} mol/l). As shown in Figure 3C, the inhibition caused by U69593 was reversed in the presence of the antagonist, suggesting action through opioid receptors. It is interesting to note that treatment of Ishikawa cells with diprenorphine alone increased TGFβ1 secretion in a statistically significant way (148.05 ± 8.48% of non-treated control, $P < 0.05$), suggesting that the antagonist blocked the action of endogenously produced opioids.

Detection of the PDYN transcript in human endometrial cells

In order to examine the expression of the *PDYN* gene by endometrial cells, preparations of total RNA from normal human secretory endometrial biopsy and Ishikawa cells, were subjected to Northern blot hybridization analysis using a *PDYN* mRNA probe. In parallel, total RNA isolated from rat pituitary and rat liver was used as positive and negative controls respectively. Figure 4A depicts the autoradiography of Northern blots revealing the presence of PDYN transcripts in RNA extracted from endometrial sources (lanes 3 and 4), the size of which was ~2.4 kb long, similar or identical to this detected in the rat pituitary (lane 1, positive control). The probe used did not hybridize with total RNA isolated from rat liver (lane 2, negative control).

Characterization and localization of dynorphin in human endometrial cells

To identify the type of immunoreactive material that is the end product of the *PDYN* mRNA expressed in endometrial cells, normal endometrial cell homogenates and culture supernatant from Ishikawa cells were concentrated and analysed by gel filtration chromatography. The bulk of immunoreactive material eluted from the chromatography exhibited the molecular weight of the 8 kDa form of dynorphins, while small quantities of a 4 kDa peptide was also detectable in culture media from Ishikawa cells (Figure 4B). It is interesting that the chromatographic profile from both sources exhibits a very similar profile, confirming the resemblance of the Ishikawa cell line behaviour to the original tissue.

Endometrial cells growing *in vitro* on slides were stained with an anti-human dynorphin antibody and examined by immunofluorescence. Ishikawa cells (Figure 5B) and epithelial cells from primary cultures (Figure 5D) were strongly positive in contrast with the negative controls (cells treated without primary antibody) (Figure 5A and C). Dynorphin was localized in the cytoplasm, where well-demarcated positive granules were observed. There was a marked concentration of immunoreactive material in the peri-nuclear area in both preparations, whereas nuclei were negative. Preparations from normal and tumoral epithelial cells revealed the same pattern of expression. Isolated stromal cells from primary endometrial cultures showed also weak cytoplasmic staining for dynorphin (Figure 5F), compared with the negative control (Figure 5E).

Discussion

Several reports suggest that opioid peptide agonists modulate the production and/or effects of growth factors in a paracrine manner. Thus, the endogenous κ-opioid agonist, dynorphin, inhibits the effect of macrophage-colony stimulating factor (M-CSF) on mouse bone marrow cells (Roy *et al.*, 1991). Similarly, the synthetic κ-opiod agonist U-69593 inhibits the proliferative effect of epidermal growth factor (EGF) on PC12 rat pheochromocytoma cells (Venihaki *et al.*, 1996). U69593 also affects the EGF-dependent modulation of extracellular signal-regulated protein kinase in monkey kidney cells (Belcheva *et al.*, 1998). μ-opioid agonists stimulate the release of TGFβ from human peripheral blood mononuclear and porcine immune cells (Chao *et al.*, 1992, Zhou *et al.*, 1992) and in rat brain they are co-localized and interact with TGF α and β (Code *et al.*, 1987; Ramirez-Ordonez *et al.*, 1999). Human endometrium is a typical example of a tissue where opioids and growth factors co-exist side-by-side. TGFβ1 represents a major endometrial growth factor (Godkin and Dore, 1998). Indeed, TGFβ1 affects the proliferation of epithelial and stromal cells of normal and tumoral human endometrium (Croxtall *et al.*, 1992; Marshburn *et al.*, 1994; Tang *et al.*, 1994, Albright and Kaufman, 1995). It should be noted that TGFβ1 also enhances the apoptosis of normal endometrial stroma (Moulton, 1994). Based on the above data we postulated that opioids may affect TGFβ1 production in human endometrium. The bulk of opioid binding sites in normal and tumoral human endometrium are of the κ1 type. Based on this data,

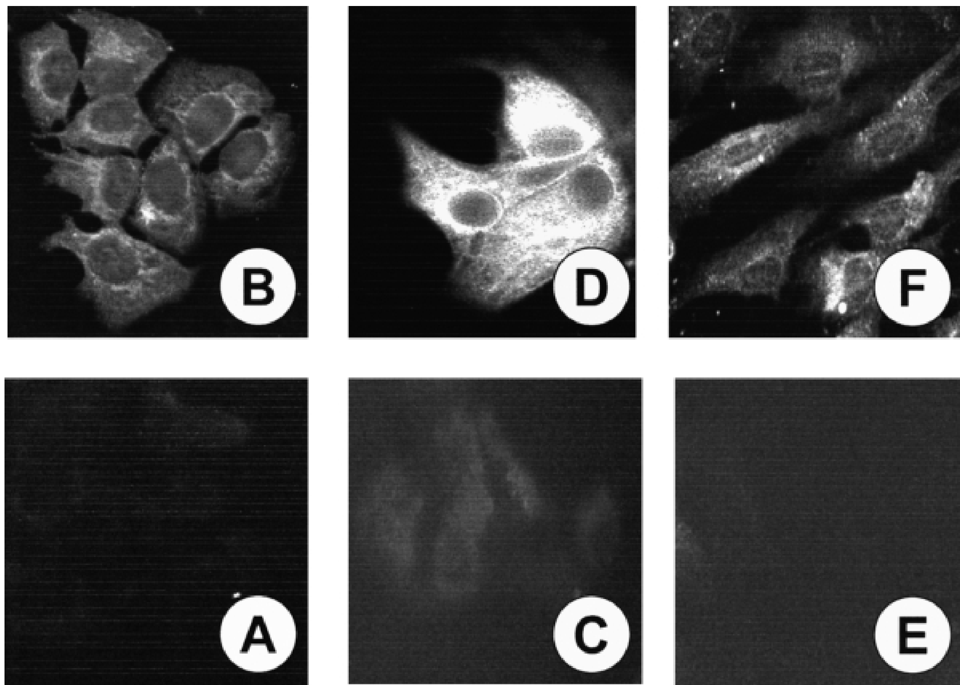

Figure 5. Immunofluorescent staining of Ishikawa (**B**), epithelial (**D**) and stromal (**F**) human endometrial cells with rabbit anti-human dynorphin serum. (**A**, **C**, **E**) Negative controls for Ishikawa, epithelial and stromal cells respectively.

we used a specific κ1-opioid receptor agonist, U69593. We have found that U69593 had a consistent inhibitory effect on TGFβ1 production from endometrial cells obtained from biopsies of proliferative and secretory endometrium. This inhibitory effect was equally demonstrable in epithelial as well as stromal cells and on the epithelial cells of the Ishikawa cell line. This effect appeared to be specifically mediated by opioid receptors since the general opioid antagonist, diprenorphine, reversed it. Interestingly, the dose–response curves of TGFβ1 were bell-shaped, showing an attenuation of this effect of U69593 at nanomolar concentrations. This type of response to opioids has been described in other systems (Yamaguchi *et al*., 1998). It may reflect the low affinity that each 'specific' opioid agonist has towards other types of opioid receptors as well as to the competition of exogenous synthetic opioids to endogenous opioid agonists, or even other endometrial factors, e.g. interleukin-6, that can activate opioid receptors (Wang *et al*., 1996). Furthermore, it has been suggested that at high concentrations, opioids may also bind to completely different receptors (Hatzoglou *et al*., 1995b). Experiments conducted using Ishikawa cells transiently transfected with the TGFβ1 promoter linked to the luciferase reporter gene have shown that treatment of the transfectants with U69593 had no effect on luciferase expression suggesting that the effect of opioids is post-transcriptional (data not shown). Our data are in agreement with reports showing that steroids and retinoids regulate TGFβ1 at a post-transcriptional level (Kim *et al*., 1992). Indeed, it has been suggested that a stem-loop which is formed in the 5′-untranslated region of the TGFβ1 transcript may represent a possible target of the κ opioids which may, thus, suppress translation (Romeo *et al*., 1993).

The paracrine effect of κ opioids on TGFβ1 in human endometrium may be part of a larger network composed of several neuropeptides regulating endometrial growth and differentiation via modulation of local growth factors. TGFβ1 appears to be such a factor regulating the proliferation of both epithelial and stromal endometrial cells as well as that of the Ishikawa cells (Croxtall *et al*., 1992; Tang *et al*., 1994; Albright and Kaufman, 1995). Furthermore, TGFβ1 stimulates stromal cell proliferation preparing the endometrium for placental implantation while, at the same time, inhibiting epithelial mitosis and promoting their differentiation (Marshburn *et al*., 1994). TGFβ1 also stimulates the apoptosis of endometrial stroma (Moulton, 1994), a mechanism responsible for the degeneration of antimesometrial decidua that enables trophoblast development and remodelling of the implantation chamber. Opioids have been shown to affect cell proliferation. Thus, it has been reported that they inhibit lung cancer (Maneckjee and Minna, 1990) and PC12 rat pheochromocytoma cell proliferation (Venihaki *et al*., 1996) while preventing their apoptosis (Dermitzaki *et al*., 2000), and in human prostate cancer cells they stimulate cell proliferation (Moon, 1988). In addition, opioid peptides inhibit the action of oestradiol on human myometrial cells (Kornyei *et al*., 1999). It is possible that at least some of these effects of opioids are mediated by locally produced growth factors affected by opioids in a paracrine manner. Indeed, in the present study, we report the expression of the main endogenous ligand for the κ opioid receptors, the dynorphin, in normal and tumoral human endometrial cells. More specifically, we have found that the *PDYN* gene transcripts and their protein end-product dynorphin are detectable in both types of cells. On Northern blot analysis the size of the transcript is 2.4 kb in size, which is similar or identical to that present in other tissues including rat pituitary and adrenals. The size of the immunoreactive material was ~8 kDa, a finding which confirmed our hypothesis. Localization

of dynorphin by immunofluorescent staining showed vesicles containing this peptide in the cytoplasm of normal and malignant endometrial cells. Epithelial cells had higher concentrations while stromal cells much less. Expression of dynorphin peptides has been previously reported in the uterus of other species. Indeed, Douglass *et al*. showed low amounts of immunoreactive dynorphin A in rat but not guinea pig and rabbit uterus (Douglass *et al*., 1987). Similarly, in human endometrial cells, we could not detect dynorphin A, while the main product of the PDYN processing was the 8 kDa bioactive peptide. Multiple forms of dynorphins are also present in another intra-uterine site, human placenta (Ahmed *et al*., 1986, 1987). However, neither the uterus nor the placenta of mice soon after implantation and at any age of gestation express dynorphins (Zhu and Pintar, 1998), implying species-specific differences.

In summary, we report here the expression of the *PDYN* gene by normal human endometrium and also the synthesis and secretion of dynorphins by these cells. In addition, we show an inhibitory effect of the κ-opioid agonist, U69593, on endometrial TGFβ1. Our results underline the significance of these neuropeptides in endometrial physiology, since they implicate their activity with the expression of a key growth factor. The cross-talk between endometrial opioids and growth factors suggest the involvement of these agents in the regulation of uterine cell proliferation and differentiation.

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