Comparisons of the effects of tamoxifen, toremifene and raloxifene on enzyme induction and gene expression in the ovariectomised rat uterus

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Abstract

This study compares the actions of oestradiol, tamoxifen, toremifene and raloxifene on enzyme and gene expression in uterine tissues of ovariectomised rats over 72 h. The time-course for the induction of ornithine decarboxylase by the compounds showed a rapid biphasic response, while for creatine kinase brain type (BB) there was a continued increase over 72 h. The efficacy of induction showed that, with both markers, oestradiol gave the highest induction level, followed by tamoxifen or toremifene and then raloxifene. RT-PCR demonstrated that all compounds decreased oestrogen receptor (ER) α , ER β and ER β 2 gene expression, 8–24 h after the first dose, suggesting that down-regulation of ER is not the primary cause of the

difference in efficacy between these compounds. Using cDNA arrays, expression of 512 genes was examined in the uteri of oestradiol- or tamoxifen-treated rats. Both compounds resulted in the up-regulation of heat-shock protein 27, telomerase-associated protein 1 and secretin. However, most surprising was the marked down-regulation of Wilms' tumour and retinoblastoma genes. We speculate that this may result in a loss of regulation of the transition from the G1 to the S phase in the cell cycle and may make cells more vulnerable to the carcinogenic effects of tamoxifen in this tissue.

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Introduction

There are a number of anti-oestrogenic drugs that have a potential use in adjuvant therapy or as chemopreventive agents for women with or at risk of developing breast cancer. Tamoxifen has already shown proven benefits as adjuvant therapy for breast cancer (Clarke *et al.* 1998). The National Surgical Adjuvant Breast Prevention P-1 study has shown an overall 49% reduction in breast cancer incidence in healthy at-risk women taking tamoxifen as a chemopreventative agent (Fisher *et al.* 1998). However, in both breast cancer patients and in healthy women there is an increase in the incidence of endometrial cancers (Fisher *et al.* 1994) and this has led to the search for safer alternatives. The structural analogue toremifene is claimed to be equally effective and well tolerated in the treatment of advanced breast cancer in postmenopausal women (Pyrhonen *et al.* 1999), although there are far fewer clinical data for this compound. Raloxifene is presently only approved for use as an anti-osteoporosis agent in postmenopausal women but, in this group, treatment resulted in a 90% decrease in the incidence of oestrogen receptor (ER)-positive breast cancers (Cummings *et al.* 1999).

Long-term dosing of rats with tamoxifen results in an increase in liver tumours (Hard *et al.* 1993, Carthew *et al.* 1995, Levenson & Jordan 1998). The increase in liver cancers has been attributed to DNA damage caused by the cytochrome P450-dependent activation of tamoxifen (White *et al.* 1992, White 1999). Toremifene and raloxifene are not known to be activated in this way. It is presently thought unlikely that initiation of uterine DNA damage plays a role in endometrial carcinogenesis in women (White 1999). If the oestrogen agonist effect of these drugs on the uterus promotes endogenous DNA damage that results in tumour formation, then actions mediated by ER α and ER β (Kuiper *et al.* 1996) may play a critical role.

We report that oestradiol, tamoxifen, toremifene and raloxifene result in a similar down-regulation of ER α , ER β and ER β 2 gene expression, determined by semi-quantitative RT-PCR. Analysis of gene expression using cDNA arrays further indicates that the genes for Wilms' tumour (WT1) and retinoblastoma proteins (Rb) are also markedly down-regulated by tamoxifen or oestradiol. We speculate that this may result in a loss of regulation of the transition from the G1 to the S phase in the cell cycle and may make cells more vulnerable to the carcinogenic effects

of tamoxifen in this tissue. An evaluation of the relative safety of these different selective ER modulators (SERMs) is important from the point of view of their use in women, particularly when employed long-term in a chemopreventive role.

Materials and Methods

Chemicals

17 β -Oestradiol benzoate, glycerol formal, tricaprilyn, ethidium bromide and creatine kinase (CK) isoenzyme kits (electrophoretic separation) were from Sigma Chemical Co., Poole, Dorset, UK. Tamoxifen citrate, toremifene citrate and raloxifene hydrochloride were gifts from Dr T C Orton, AstraZeneca, Macclesfield, Cheshire, UK. Oligo dt₁₂₋₁₈ primer, dNTPs, Superscript II RNase H-reverse transcriptase, RNase and agarose were from Life Technologies, Paisley, Strathclyde, UK. *AmpliTaq* DNA polymerase and oligonucleotide primers were from Perkin Elmer, Warrington, Cheshire, UK. DNase I (RNase free) was from Promega, Southampton, Hants, UK. [1-¹⁴C]Ornithine (specific activity 2.07 GBq/mmol) and I [α -³³P]dCTP (specific activity 92.5 TBq/mmol) were from Amersham International plc, Amersham, Bucks, UK.

Animals and treatment

Groups of six ovariectomised Wistar (Han) rats of 9 weeks of age (Charles River Ltd, Margate, Kent, UK) were housed in negative pressure isolators with a 24-h light:darkness cycle and allowed free access to food and water. The study was conducted under the authority of the United Kingdom Home Office, Animals (Scientific Procedures) Act 1986. 17 β -Oestradiol benzoate (0.133 μ mol/kg, tamoxifen citrate, toremifene citrate or raloxifene hydrochloride (all at 1.88 μ mol/kg) in glycerol formal were administered subcutaneously once a day for 3 days. Control animals received vehicle only. Rats were killed at 1, 2, 4, 6, 8, 10, 12, 15, 24, 48 and 72 h after the first dose. For dose-response studies, drugs were given subcutaneously as a single dose. Treatments used were oestradiol at 0.026, 0.53, 0.133, 0.265, 0.532 μ mol/kg or tamoxifen citrate, toremifene citrate or raloxifene hydrochloride at 0.53, 0.94, 1.88, 3.76, 7.52 μ mol/kg. In all instances, animals were killed by phenobarbitol overdose and uterine tissues quickly removed, weighed and snap frozen in liquid nitrogen. In some instances, representative sections of both horns of the uterus were fixed in 10% neutral buffered formalin for immunohistochemistry.

Ornithine decarboxylase activity

Activity of ornithine decarboxylase (ODC) was measured in uterine cytosolic fractions by the conversion of

[1-¹⁴C]ornithine to [¹⁴C]CO₂ (Olson *et al.* 1983, Tanaka *et al.* 1993). Uterine homogenates were prepared in 10% (w/v) ice-cold 100 mM sodium phosphate buffer (pH 7.2) containing 5 mM dithiothreitol and centrifuged at 100 000 g for 50 min at 4 °C. The protein content of the supernatant was determined by the method of Lowry *et al.* (1951).

Creatine kinase BB activity

Activities of CK-BB in uterine cytosolic fractions, prepared as described above, were determined following agarose gel electrophoresis, according to the manufacturer's instructions. CK-BB isoenzyme (Calbiochem, Nottingham, Notts, UK) standards were included on each gel. The density of the bands was determined using a Molecular Dynamics densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Cell culture and transfections

Human uterine endometrial derived HEC-1-A cells were maintained and transfected as previously described (Jones *et al.* 1999). Reporter plasmid PAM119 *ckb*-Luc was created by cloning the 2.9 kb *Hind*III fragment of *ckb*-CAT (Somjen *et al.* 1996) into the *Hind*III site of pGL3 Basic (Promega). Expression plasmids were mER α -pJ3 MOR (White *et al.* 1987), mER β -pCNX2-mER β (Tremblay *et al.* 1997) and rER β -pCDNA-ER β -6 (Petersen *et al.* 1998). The internal control plasmid was pCMV β (Clontech, Basingstoke, Hants, UK). Each plasmid was used at a concentration of 0.25 μ g/plasmid. Cells were dosed 4 h after transfection in triplicate with 17 β -oestradiol (10⁻⁸ M), tamoxifen, toremifene, raloxifene (all at 10⁻⁶ M) or dimethyl sulfoxide vehicle control. Cells were harvested at 24 h and analysed for β -galactosidase and luciferase activity using detection kits (Promega).

Total RNA extraction

Snap-frozen rat uterine tissue was ground to a fine powder in a pre-chilled sterile mortar and pestle in liquid nitrogen. Total RNA was extracted using the method of Chomczynski & Sacchi (1987). To digest contaminating DNA, the RNA preparation was incubated with DNase I following the manufacturer's instructions and further purified by phenol/chloroform extraction. RNA levels were determined from their A₂₆₀ (absorbance at 260 nm) values. RNA preparations were stored at -80 °C.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR for genes encoding ER α , ER β , ER β 2, ODC, heat shock protein (HSP) 27, Rb,

Table 1 Oligonucleotide primers for RT-PCR

Gene target	Oligonucleotide primers (5'–3')	Annealing temperature (°C)	PCR cycles
ER α	GTTTGCTCCTAACTTGCTCTTGGACAGG TCCAGCATCTCCAGCAGCAGGTC	60	26
ER β /ER β 2	CTCACTAAGCTGGCGGACAA GCAGAAGCCAAGGGGTACAT	50	36
GAPdh	TGAAGGTCGGTGTCAACGGATTT CACAGTCTTCTGAGTGGCAGTGAT	55	22
ODC	ATGTCATTGGTGTGAGCTTCC GTGAAAGCTGATGCGACGTA	50	26
HSP27	GAAATACACGCTCCCTCCAG GGCTTCTACTTGGCTCCAGA	60	26
Rb	GCCCAGCAGTGGGTTATC CTTTCAAGCTCTGGAAGTCCA	50	26
Secretin	AGCGAGGAGGACACAGAAAA CAAAGGACAACCAATCCCTA	55	30
WT1	CGGCTGTGCATCTGTAAGTG GAGCTACCTGAAGGGAATGG	50	26

secretin and WT1 were determined in the same sample and normalised against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPdh). For each sample, a template of 0.5–1.0 μ g total RNA was used in cDNA synthesis using oligo dt_{12–18} primer and Superscript II RNase H-reverse transcriptase, according to the manufacturer's instructions. Expression of each gene was amplified in duplicate in a total volume of 20 μ l using 0.5 U *AmpliTaq* DNA polymerase, 0.2 mM dNTPs, 0.2 μ M each sense/antisense primer (Table 1) in thin-walled microfuge tubes and overlaid with mineral oil. The following PCR incubation times were used: 94 °C for 5 min followed by cycles of 94 °C for 30 s, annealing temperature (Table 1) for 30 s, 72 °C for 30 s and concluded by 72 °C for 5 min. The number of cycles for each gene target was previously determined so that amplification was in the linear phase (Table 1; data not shown). A negative control, where water was substituted for cDNA, was included in each PCR experiment. The resulting PCR products for each sample were electrophoresed in parallel through a 2% agarose gel with 5 ng/ml ethidium bromide and visualised under UV. The densities of the bands were determined using densitometry (Molecular Dynamics; ImageQuant software) and in each instance normalised against the density of the corresponding GAPdh PCR product. Representative PCR products were verified by DNA sequencing (data not shown).

cDNA microarrays

Total RNA was extracted and pooled from four separate uterine samples of ovariectomised rats treated with

oestradiol (0.133 μ mol/kg), tamoxifen (1.88 μ mol/kg) or vehicle as described above. DNase-treated total RNA (15 μ g) was used as a template for the preparation of radiolabelled cDNA probes using Superscript II RNase H-reverse transcriptase and [α -³³P]dCTP. The cDNA microarray nylon filter was created within Safety Assessment, AstraZeneca, UK and consisted of a total of 512 mouse genes, each represented by two separate clones in duplicate. The gene categories included apoptosis, cell–cell communication, cell cycle, nuclear receptors, transcription factors, cytokines/chemokines, drug metabolism, stress response, DNA repair, steroidogenesis and steroid-regulated genes. The clones were identified from the NIH public domain and printed onto filters using a Q Bot (Genetix, Christchurch, Dorset, UK). Following incubation at 42 °C for 2 h, reactions were terminated by heating at 70 °C for 15 min. RNA template was digested by 1 μ l RNase at 37 °C for 20 min. The unincorporated label was removed using ProbeQuant G-50 micro columns (Amersham Pharmacia Biotech, St Albans, Herts, UK) following the manufacturer's instructions. Incorporation of radiolabel for each probe was determined and adjusted accordingly. Microarray filters were incubated in Church hybridisation solution (0.5 M sodium phosphate buffer (pH 7.2), 7% SDS, 1 mM EDTA; Sigma) at 65 °C for 1 h prior to addition of probe (boiled for 5 min and ice quenched) in fresh hybridisation solution. The filter was incubated at 65 °C for 18 h and rinsed twice in Church wash solution (40 mM Church sodium phosphate buffer, 1% SDS; Sigma) at 65 °C for 20 min. The filter was exposed to phosphorimager film overnight and image analysis of hybridisation densities was assessed using Array Vision image analysis software (Molecular Dynamics).

Hybridisation with each pooled RNA sample was carried out in triplicate.

Immunohistochemistry

For immunohistochemical localisation of ER α and ER β , 5 μ M neutral buffered formalin-fixed uterine sections were dewaxed with xylene, taken to water and micro-waved in citrate buffer (pH 6.0) for 20 min at 700 W power. Sections were placed in distilled water at room temperature and endogenous peroxidase activity blocked with 3% (v/v) H₂O₂ in water. For ER α , a monoclonal anti-human antibody, raised against a prokaryotic protein corresponding to the full-length ER α , was used at a dilution of 1:40 (NCL-ER-6F11; Novacastra Laboratories Ltd, Newcastle-Upon-Tyne, Tyne and Wear, UK). For ER β , a polyclonal rabbit antibody, raised against the peptide residues 54–71 of rat ER β , was used at a dilution of 1:10 (ID 06629; Upstate Biotechnology, New York, NY, USA). Immunoreactive nuclei were visualised with 3,3'-diaminobenzidine/H₂O₂. Sections were lightly counterstained with haematoxylin. The specificity of antibody staining was assessed by titrating the antigen-specific peptide recommended by the manufacturers with the primary antibody. Additional sections were stained using standard haematoxylin and eosin stains for histological analysis.

Statistical analysis

Statistical analyses were carried out using Minitab version 10 (Minitab Inc., State College, PA, USA). Differences between groups were tested using analysis of variance (ANOVAR) with Dunnett's test for significance at the 5% level.

Results

Time-dependent regulation of ODC and CK-BB by SERMs

The induction of ODC and CK-BB by oestradiol correlates with an increase in uterine wet weight in ovariectomised rats and provides useful markers of oestrogenicity (Olson *et al.* 1983, Somjen *et al.* 1996, Hibasami *et al.* 1999). The effects of SERMs and oestradiol on the activities of these enzymes were therefore compared. Treatment of ovariectomised rats with oestradiol resulted in a biphasic induction of uterine ODC activity with peaks at 6 and 24 h (Fig. 1A), confirming earlier studies with this steroid (Branham *et al.* 1988). Dosing with tamoxifen or toremifene resulted in similar time-courses of induction, but the effects were less marked than seen with oestradiol (Fig. 1C–D). The induction of ODC activity associated with raloxifene treatment was even weaker (Fig. 1B).

Figure 1 Time- and dose-dependent regulation of ODC by oestradiol, tamoxifen, toremifene or raloxifene in the ovariectomised rat uterus. ODC activities were determined in uterine cytosolic fractions from rats at the times indicated after administration at 0, 24 and 48 h of (A) oestradiol (0.133 μ mol/kg), (B) raloxifene, (C) tamoxifen or (D) toremifene (all at 1.88 μ mol/kg). (E) The dose-response for ODC activity at 24 h after administration of oestradiol (0.026–0.532 μ mol/kg) ($\square$), raloxifene ($\circ$), tamoxifen ($\triangle$) or toremifene (∇) (all at 0.53–7.52 μ mol/kg). Glycerol formal vehicle was used in controls. Each value represents the means $\pm$ s.e. for at least four animals. * P < 0.05 compared with vehicle-treated controls.

Repeated administration of the drug at 24 and 48 h did not result in further induction with any of the compounds. With all treatments, ODC activity returned to basal levels by 72 h. Dose-response curves for ODC activities are shown in Fig. 1E. Increasing the dose of raloxifene did not increase the level of enzyme activity to that caused by equivalent doses of tamoxifen or toremifene. The levels

Figure 2 Time-dependent regulation of CK-BB in the ovariectomised rat uterus by oestradiol or SERMs. CK activities were measured in uterine cytosolic fractions prepared from ovariectomised rats at the times indicated after administration at 0, 24 and 48 h of (A) oestradiol (0.133 $\mu\text{mol/kg}$), (B) raloxifene, (C) tamoxifen or (D) toremifene (all at 1.88 $\mu\text{mol/kg}$). Glycerol formal vehicle was used in controls. Each value represents the means $\pm$ S.E. for four animals. * $P < 0.05$ compared with vehicle-treated controls.

of induction of ODC mRNA at 24 h, relative to controls, were $161 \pm 12\%$, $131 \pm 3\%$ and $107 \pm 8\%$ (means $\pm$ S.E.M., $n=4$) for tamoxifen, toremifene and raloxifene respectively when compared at 1.88 $\mu\text{mol/kg}$.

Uterine CK-BB activities, in contrast, continued to rise throughout the 72-h treatment (Fig. 2). The efficacy of induction with the different SERMs was similar to that seen using ODC with oestradiol being the best inducer (Fig. 2A), followed by tamoxifen or toremifene (Fig. 2C–D) and then raloxifene (Fig. 2B). Electrophoretic separation of the CK isoenzymes showed the presence only of the BB form in the uterine extracts. To investigate the role of ER subtypes in the activation of CK-BB, a human-derived uterine cell line HEC-1-A (effectively ER silent) was co-transfected with *ckb*-Luc promoter construct and mouse ER α , ER β or rat ER β 2. Figure 3 shows that oestradiol, tamoxifen and toremifene resulted in a significant activation ($P < 0.05$, ANOVA) of the *ckb*-Luc promoter in the presence of ER α compared with controls. Only oestradiol activated the *ckb*-Luc promoter in the presence of ER β . No response was seen in the presence of ER β 2 irrespective of compound. There was no effect of raloxifene on the *ckb*-Luc promoter with any of the ER subtypes. None of the compounds had any significant effect on HEC-1-A cells not cotransfected with ER constructs.

Figure 3 Activation of the *ckb*-Luc reporter construct in HEC-1-A cells by oestradiol or SERMs. Monolayer cultures of HEC-1-A cells were co-transfected with *ckb*-Luc (0.25 g) together with an expression plasmid for ER α , ER β or ER β 2 (0.25 g). Cells were treated 4 h after transfection in triplicate with oestradiol (10^{-8}M), tamoxifen, toremifene or raloxifene (all at 10^{-6}M) or vehicle control. Cells were harvested at 24 h and analysed for luciferase activity. Each value represents the means $\pm$ S.E. of each group. * $P < 0.05$ compared with vehicle-treated controls.

Time- and dose-dependent regulation of ER

The effects of oestradiol, tamoxifen, toremifene or raloxifene on mRNA encoding ER α , ER β and ER β 2 in the uterine tissues was investigated. Figure 4 shows that oestradiol, tamoxifen, toremifene and raloxifene all decreased ER α (Fig. 4A), ER β (Fig. 4B) and ER β 2 (Fig. 4C) gene expression over the 72-h dosing period. Particularly when comparing the levels of ER β and ER β 2, there was evidence for parallel regulation. Maximum down-regulation, three- to ninefold below control values, occurred 8–24 h after the first dose. In spite of repeated dosing at 24-h intervals, all three receptors started to return to control levels after 24 h in the tamoxifen-, toremifene- and raloxifene-treated animals. After 72 h, ER β and ER β 2 remained more strongly down-regulated by raloxifene compared with tamoxifen or toremifene.

Gene microarray analysis

Figure 5 shows two log-log scatter plots using cDNA arrays. Pairwise comparisons of cDNA hybridisations were made between pooled uterine RNA samples of controls and either oestradiol (Fig. 5A) or tamoxifen (Fig. 5B). Each gene of the 512 genes on the array is represented by a point on the scatter plots and the density of each gene was normalised to the sum of the density of all genes. The hybridisation signal for each gene analysed three times was comparable (data not shown). Comparing expression between oestradiol and control, 94 genes (18%) were up-regulated and 41 genes (8%) down-regulated greater than twofold. Tamoxifen treatment resulted in a twofold or greater up-regulation of 149 genes (29%) and down-regulation of 15 genes (3%). Of these genes, only 6 (1%)

Figure 4 Time-dependent gene regulation of ER mRNA expression by oestradiol and SERMs in the ovariectomised rat uterus. mRNA encoding (A) ER α , (B) ER β and (C) ER β 2 were determined using semi-quantitative RT-PCR, normalised to the GAPdh housekeeping gene, at the times indicated after administration at 0, 24 and 48 h of oestradiol (E2; 0.133 μ mol/kg), raloxifene, tamoxifen or toremifene (all at 1.88 μ mol/kg). Glycerol formal vehicle was used in controls. Each value represents the means $\pm$ s.e. for three animals.

Figure 5 Differential expression of genes by oestradiol (E2) and tamoxifen in the ovariectomised rat uterus using cDNA microarray analysis. The levels of gene expression were determined in pooled uteri as described in Materials and Methods at 24 h after administration of (A) oestradiol (0.133 μ mol/kg), (B) tamoxifen (1.88 μ mol/kg) or vehicle alone as control. Each point represents the mean expression of a gene transcript, adrenergic receptor (α_{1a} AR), chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), HSP27, Rb, secretin (Sec), telomerase-associated protein 1 (TEP1) and WT1.

Table 2 Genes regulated by oestradiol (E2) and tamoxifen revealed by gene array analysis together with known functions

Gene	Fold change ^a		Putative function
	E2	Tamoxifen	
α_{1a} AR	+12	+44	Mediates contractions in myometrium of uterus and is regulated by E2 in rabbit uterus (Riemer <i>et al.</i> 1987)
HSP27	+5	+9	Involved with cell proliferation of endometrium and is up-regulated during endometrial and breast carcinogenesis (Cano <i>et al.</i> 1986, Devaja <i>et al.</i> 1997)
TEP1	+2	+3	Required for telomerase activity which is activated by oestrogen directly and indirectly (Ito <i>et al.</i> 1998, Kyo <i>et al.</i> 1999). In MCF-7 breast cancer cells it is not particularly associated with acquired resistance to tamoxifen or oestradiol (Hilsenbeck <i>et al.</i> 1999).
Secretin	+6	+29	Expressed in cells of the diffuse neuroendocrine system, where it stimulates the secretion of both H^+ and HCO_3^- ions, the GI tract and in bile ductules (Villanger <i>et al.</i> 1992, Chang <i>et al.</i> 1998)
Rb	-8	-7	Regulates the progression of cells from G1 to S phase and the subsequent progression of cells through S phase (Zhang <i>et al.</i> 2000)
WT1	-33	-35	Tumour-suppressor gene and critical regulator of cell proliferation and differentiation (Smith <i>et al.</i> 2000)
COUP-TFII	-74	-61	Orphan nuclear hormone receptor which can act as a repressor or activator of transcription via interaction with ER (Qiu <i>et al.</i> 1996, Klinge <i>et al.</i> 1997, Petit <i>et al.</i> 1999)

^aFold induction (positive numbers) and reduction (negative numbers) compared with gene expression in control uterus.

α_{1a} AR, gene transcript adrenergic receptor; TEP1, telomerase-associated protein 1; COUP-TFII, chicken ovalbumin upstream promoter transcription factor II; GI, gastrointestinal tract.

were regulated by tamoxifen alone and these were all up-regulated. Several genes of interest, and their putative functions, which were up- or down-regulated by oestradiol and tamoxifen compared with the control by more than twofold are listed in Table 2.

The validity and reliability of the cDNA microarray analysis was confirmed using semi-quantitative RT-PCR using the same samples as for hybridisation. Products were identified by DNA sequencing and corresponded to expected gene sequences. Changes in expression for HSP27, ODC, Rb, secretin and WT1 genes were comparable to those observed in the cDNA microarray (data not shown).

Cellular localisation of ER

Histological examination of the uterus from control rats showed a condensed myometrium and endometrial stroma. The endometrial epithelium was predominantly cuboidal. Compared with controls, the uteri from rats treated with tamoxifen showed expansion of the myometrium and endometrial stroma as described previously (Carthew *et al.* 1999). Compared with controls (Fig. 6A and B), the epithelium was high columnar in type and it contained enlarged nuclei showing mild pseudostratification following tamoxifen treatment (Fig. 6C and D). As uterine tissue is comprised of a number of different cell types, localisation of ER was determined by immunohistochemistry. ER α was localised in both the glandular and luminal epithelial cells and the myometrium (Fig. 6A) confirming earlier studies (Carthew *et al.* 1999). The localisation of uterine ER β has not previously been described; we found it to be expressed in a similar

distribution to that of ER α (Fig. 6B). In stroma cells and myocytes, labelling was predominantly nuclear in distribution. Increased labelling of ER β was apparent in stromal cells in tamoxifen-treated animals (Fig. 6D). ER α and ER β labelling of endometrial epithelial cells was intense in controls where it appeared to be both cytoplasmic and nuclear in distribution (Fig. 6A and B). In treated rats, this staining was attenuated, although nuclear staining for ER α (Fig. 6C) and ER β (Fig. 6D) remained prominent. Similar results described for tamoxifen were observed after toremifene treatment; raloxifene had a much weaker effect (data not shown). As the ER α was a monoclonal antibody and the ER β antibody polyclonal, it was difficult to make any direct comparison in terms of level of staining. It can be concluded, however, that both antibodies stain similar cell types.

Discussion

While tamoxifen is effective in preventing breast cancer, its use has been associated with an increase in endometrial cancer (Fisher *et al.* 1994, 1998, Rutqvist *et al.* 1995). It therefore a concern that other SERMs, such as toremifene and raloxifene, might have tissue-specific oestrogenic actions in the uterus similar to those seen with tamoxifen. The predominant effect at 24 h of oestradiol, tamoxifen and toremifene in ovariectomised rats is hypertrophy of uterine cells. At this time, there are no effects on cell proliferation in the luminal or glandular epithelium and it only increased in the stroma (Carthew *et al.* 1999). However, changes in gene expression must precede cell proliferation. We have shown that although there are

Figure 6 Immunohistochemical detection of ER α and ER β in the ovariectomised rat uterus. Formalin-fixed sections from the uteri of ovariectomised control rats (A and B) or animals treated with tamoxifen (1.88 μ mol/kg for 3 days) (C and D) were subjected to immunohistochemistry with specific antibodies to ER α (A and C) or to ER β (B and D) as described in Materials and Methods. Magnification for all sections was $\times 820$. The hyperplasia and hypertrophy in all cell types due to tamoxifen treatment (C and D) compared with controls (A and B) is clearly evident.

differences in the magnitude of the response with oestradiol, tamoxifen and toremifene, all had qualitatively similar effects on the uterus in inducing ODC and CK-BB and down-regulating ER α and ER β mRNA expression. The CK-BB promoter sequence has limited sequence similarity to the consensus oestrogen-response element (ERE) (Bergen *et al.* 1993). The present transfection studies with CK-BB promoter show there to be clear differences between the SERMs studied in regulating gene expression – particularly that oestradiol acts through both ER α and ER β , while tamoxifen and toremifene act only via ER α . Raloxifene, which also induced CK-BB in uterine tissues, was not active in this gene reporter system. The results suggest that the induction of this enzyme activity may be partially mediated by ER-independent mechanisms (Wu-Peng *et al.* 1992). ODC gene expression does not reflect enzyme activity in response to SERMs and therefore suggests the regulation of its activity via post-translational control more than those through classical ERE (Kameji *et al.* 1993).

In such a heterogeneous tissue as the uterus, the most significant limitation of the present gene expression study is to accurately reflect events occurring in different cell types. For example, down-regulation of ER α protein occurs in the luminal epithelial cells with oestradiol treatment but not with tamoxifen or toremifene (Carthew *et al.* 1999). The epithelium represents 2–3% of total uterine RNA (McCormack & Glasser 1980) and therefore specific changes in the epithelial ER could be considerably attenuated in whole tissue extracts. Immunohistochemistry has been used to demonstrate that ER α and ER β are expressed in the ovariectomised rat uterus, similar to previous studies (Saunders *et al.* 1997, Carthew *et al.* 1999).

cDNA microarray systems have been used to analyse changes in expression of large numbers of genes in response to oestradiol and tamoxifen and identify novel genes not normally associated with uterine cells. In ovariectomised rats, oestradiol and tamoxifen act as oestrogen agonists (Carthew *et al.* 1999) but it is remarkable that the

overall change in the pattern of gene expression after 24 h is so comparable. Of the genes that were down-regulated by both oestradiol and tamoxifen, especially marked were those for WT1 and Rb proteins. Down-regulation of these genes in uterine cells may make them more susceptible to neoplasia, although the factors that differentiate oestradiol relative to tamoxifen have yet to be established.

Of the up-regulated genes, cDNA arrays have shown the HSP27 gene to be up-regulated by tamoxifen or oestradiol. Previous studies using MCF-7 breast cancer cells have shown this gene to be the most highly expressed, but not particularly associated with the acquired resistance of this cell line to tamoxifen or oestradiol (Hilsenbeck *et al.* 1999). The present study shows high expression of the gene for the telomerase component, telomerase-associated protein 1 and its up-regulation by tamoxifen and oestradiol in uterine tissues. Somewhat unexpectedly, we found secretin gene expression in the uterus after up-regulation by either oestradiol or tamoxifen. As far as we are aware, secretin is a novel gene for uterine tissues classically found in cell types of the gastrointestinal tract. Other studies have used subtractive cDNA libraries to examine oestrogen-induced genes in the uterus of ovariectomised rat, 4 h after dosing with oestradiol, tamoxifen or droloxifene (Rivera-Gonzalez *et al.* 1998). Up-regulation of eight genes that included the interleukin 4 receptor, HSP70 and metallothionein was found but none of these genes was identified in the present study.

Our results show that over 72 h, the down-regulation of both ER receptors by oestradiol, tamoxifen, toremifene and raloxifene is similar. However, with respect to enzyme induction, tamoxifen and toremifene have similar effects while raloxifene is a weaker oestrogen agonist. The use of cDNA microarrays has provided a partial insight into the complex patterns of gene expression and their changes in the rat uterus in response to oestradiol and tamoxifen.

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