The molecular basis of tamoxifen induction of mouse uterine epithelial cell proliferation

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Abstract

Tamoxifen, a selective estrogen modulator (SERM) that has found clinical utility in the treatment of breast cancer, is an antagonist in the breast and an agonist in the uterus. These agonist actions in the uterus lead to an increased risk of endometrial cancer. In this study in mice we have analyzed the mechanism of action of tamoxifen in inducing cell proliferation in the uterine luminal epithelia. Tamoxifen induces a wave of DNA synthesis in these epithelial cells with kinetics similar to those seen after 17β -estradiol (E_2) treatment. However, by these criteria of mitogenicity, it is much less potent and never achieves full estrogenicity. This uterine epithelial cell proliferation is preceded by the mobilization of cyclin D1 from the cytoplasm to the nucleus which, together with CDK4, phosphorylates members of the Rb-retinoblastoma family of proteins, pRb and p107. Subsequent to this initial nuclear accumulation of cyclin D1, cyclin E and then

cyclin A are induced that, together with the activation of CDK2, results in enhanced cyclin E- and cyclin A-dependent CDK2 kinase activity and further phosphorylation of pRb and p107. These actions of tamoxifen parallel those of E_2 . Tamoxifen also induced the classical estrogen water imbibition response. However, in this it was more potent, producing a maximal response at doses that do not affect DNA synthesis. This suggests that the uterotrophic response is not an accurate predictor of the compound's hyperplasia responses. We can conclude that, in its effects on proliferation, tamoxifen acts as a classical impeded estrogen and this suggests that the AF-1 transcription activation domain of the estrogen receptor that is activated upon both E_2 and tamoxifen binding to this receptor regulates these responses in the uterus.

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Introduction

Estrogen induces cell proliferation in the uterine and mammary gland epithelium (Martin *et al.* 1973, Tong & Pollard 2002). Exposure to estrogen is also the major risk factor in the development of adenocarcinomas of these organs (Travis & Key 2003). Indeed, most of these tumors are hormone dependent for their growth before progressing to hormone independence. Consequently, endocrine ablation either by removal of the ovary or by administration of anti-estrogenic drugs has been a major therapeutic option. However, removal of estrogen has a negative impact on women's health, increasing the risk for osteoporosis and cardiovascular disease (Jordan & Morrow 1999). Recently, a group of drugs have been developed that have selective effects on hormone responsive tissue and therefore are known as selective estrogen receptor response modulators or SERMs (Jordan & Morrow 1999, McDonnell 1999). The most successful of them to date is tamoxifen. In large clinical trials this drug was shown to have a very significant positive impact upon disease-free

survival rates in breast cancer patients because it acts as an estrogen antagonist in the mammary epithelium (Jordan & Morrow 1999). In humans, tamoxifen is an agonist in other estrogen-responsive tissues including the uterus, where it stimulates uterine epithelial cell proliferation (Ismail 1996, Baum 1997, Jordan & Morrow 1999). This effect upon the endometrium unfortunately results in an increased risk of endometrial cancer in patients taking the drug (Bergman *et al.* 2000).

It has been shown by studies, mostly in tissue culture cells, that the duration of the cell cycle is regulated by the binding of the regulatory cyclins with their cyclin-dependent kinase (CDK) partners (Sherr 1994). Positive activation of CDK involves assembly with their cyclin partners and phosphorylation by cyclin activating kinase. On the other hand, interaction with CDK inhibitors (CDI) negatively regulates their activity (Sherr 1996, Sherr & Roberts 1999). The active CDKs phosphorylate their nuclear protein substrates, members of the retinoblastoma (pRb) family of pocket proteins, which, in turn, alleviates the negative control of these proteins over certain

transcription factors, such as the E2Fs, required for cell cycle progression (Ewen *et al.* 1993, Sherr 1996, Sherr & Roberts 1999). Different cyclins act sequentially through G₁, with members of the cyclin D family activating CDK4 and CDK6 first, followed by cyclin E/CDK2 at the G₁/S phase transition and cyclin A/CDK2 throughout S phase (Ohtsubo & Roberts 1993, Sherr 1994). In this scenario the D-type cyclins are considered to be the sensors of external signals, modulating the rate of cell division (Sherr 1994).

The high potency natural estrogen, estradiol-17 β (E₂), when given to ovariectomized mice results in a synchronized wave of DNA synthesis in the luminal and glandular epithelium of the uterus beginning at 6 h and peaking at 12–15 h after administration, followed by a wave of cell division and re-entry into another cell cycle (Finn & Martin 1970, Kaye *et al.* 1972, Martin *et al.* 1973, 1976, Tong & Pollard 2002). Recently, we have shown that E₂ controls the uterine epithelial cell cycle through the activation of cyclin dependent kinases which resulted in hyperphosphorylation of pRb and p107 and subsequently enhanced cell cycle progression (Tong & Pollard 1999). Estrogen treatment of breast carcinoma cells results in a dramatic elevation of cyclin D1 mRNA and protein (Matsushima *et al.* 1991, 1994, Musgrove *et al.* 1993, 1994, Prall *et al.* 1997). However, in uterine epithelial cells *in vivo* there was only a moderate induction of cyclin D1 by E₂ and most of this was at a later stage than the phosphorylation of pRb (Tong & Pollard 1999). Instead, E₂ mobilized cyclin D1 and to a lesser extent CDK4 from the cytoplasm to the nucleus, thus giving the active kinase complex access to its nuclear localized pRb family of substrates (Tong & Pollard 1999). These data suggest that the mobilization of cyclin D1 into the nucleus is a central regulatory point for estrogen action in the uterus.

In the mouse uterus, tamoxifen is an agonist inducing epithelial cell proliferation while showing little to no antagonistic effect in the presence of E₂ (Martin & Middleton 1978, Klotz *et al.* 2000). Long-term administration induces persistent changes in the uterus including cystic hyperplasia in the glands (Martin & Middleton 1978). This contrasts with the rat where tamoxifen was as effective as ovariectomy in reducing proliferating cell nuclear antigen (PCNA) expression, a marker for cell proliferation, although the effects were somewhat cell type specific (Rumpel *et al.* 1995), and in chickens where it is fully antagonistic without estrogenic properties (Sutherland *et al.* 1977). Humans appear to be more similar to mice than to rats in their uterine responses, since tamoxifen acts agonistically stimulating cell proliferation (Ismail 1996, Jordan & Morrow 1999, Willen *et al.* 2002). These differences could be mediated through different metabolic routes. However, there have been no species-specific variations in estrogen metabolism identified to date. This has led to the concept of target site-specific regulation and many have focused upon differential

estrogen receptor (ER) modulation by tamoxifen (Jordan & Morrow 1999).

There have been few studies on the molecular basis of this agonistic action in the uterus. Some have analyzed the induction of specific estrogen-responsive genes that might affect cell proliferation such as transforming growth factor- β 1, vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF)-I/IGF binding proteins (Hung & Pollak 1995, Sartor *et al.* 1995, Hyder *et al.* 1996, Klotz *et al.* 2000, Mueller *et al.* 2003), but none have specifically addressed the control of the cell cycle machinery in uterine epithelial cells with the exception of some studies on cyclin D expression (Geum *et al.* 1997). However, in cancer cells in culture there have been several studies that have explored the antagonistic effect of tamoxifen (Watts *et al.* 1994, Planas-Silva & Weinberg 1997), although these actions have not necessarily been dependent upon the ER (Lee *et al.* 2000). A central mechanism appears to be through the induction of the CDIs, p21^{WAF1} and p27^{KIP1}, combined with a reduction in cyclin D1 protein that acts to inhibit cyclin D/CDK4 and cyclin E/CDK2 activity and arrest cell cycle progression (Wilcken *et al.* 1997, Lee *et al.* 1999, 2000, Doisneau-Sixou *et al.* 2003). Interestingly, over-expression of cyclin D1 (Wilcken *et al.* 1997) or cyclin E can alleviate tamoxifen inhibition of MCF-7 cells in culture (Dhillon & Mudryj 2002). In some cases cyclin E can also replace cyclin D1 function in regulating the cell cycle (Lukas *et al.* 1997, Geng *et al.* 1999). Furthermore, over-expression of cyclin E can stimulate cell cycle transit without influencing cyclin D1/CDK4 activity or pRb phosphorylation (Lukas *et al.* 1997). Since cyclin D1 appears to be a major point of control for E₂ regulation of the uterine epithelium, then an explanation for tamoxifen's agonist role without fully activating the estrogen receptor may be that tamoxifen exerts its action in a manner different from that of E₂ through regulating other components of the cell cycle such as cyclin E. Given the diverse receptor- and non-receptor-mediated effects of tamoxifen and its importance in breast cancer chemotherapy in humans, we analyzed the proliferation response to tamoxifen in the mouse uterine epithelial cells *in vivo* and its effects on the cyclin-dependent cell cycle machinery in these cells. We found that it is an impeded estrogen with a $\approx 200\,000$ -fold lower potency than E₂ but that it exerts its effects on cell proliferation in a manner similar to E₂.

Materials and Methods

Animals and treatment

Adult female CD1 mice (Charles River, Wilmington, MA, USA), maintained in a 12-h light:12-h darkness cycle and weighing 25–30 g, were ovariectomized, rested for two weeks, followed by two days of priming with E₂ as described (Tong & Pollard 1999). Six days later, groups

of 3–5 mice were given one of the following treatments: (i) peanut oil, (ii) 50 ng E₂, (iii) a range of concentrations (0.125–1.5 mg/mouse) of tamoxifen. All hormones were purchased from Sigma and administered subcutaneously in peanut oil.

At various times after hormone treatment mice were killed, uteri removed and weighed, and either processed for immunohistochemistry or the luminal epithelium was removed for biochemical analysis as described previously (Tong & Pollard 1999). In some cases, to assess the percentage of cells in S-phase, BrdU was given intraperitoneally 2 h before killing as described by Tong & Pollard (1999).

Immunohistochemistry

Uteri were fixed overnight in Bouin's solution or peroxide–lysine–2% paraformaldehyde–0.5% glutaldehyde (PLPG) and processed for paraffin embedding. Transverse sections (5 µm) through the mid-point of the uterus were prepared and immunostained for BrdU incorporation, or for PCNA, cyclin A, CDK2, CDK4 or cyclin D1 protein as described (Tong & Pollard 1999). Polyclonal antibodies to CDK2 (sc-163), CDK4 (sc-260), p107 (sc-318) and cyclin A (sc-596) were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA and validated by peptide inhibition and titration as described (Tong & Pollard 1999). Monoclonal antibodies to pRb (G3–245) were obtained from Pharmingen (San Diego, CA, USA), for cyclin D1 (DCS-6) they were obtained from Neomarkers (Freemont, CA, USA) and for PCNA (PC10) they were obtained from Boehringer Mannheim. Controls also included incubation in normal serum corresponding to the source of the antibody used and omission of the primary antibody. These were uniformly negative. To determine the percentage of nuclear positive luminal and glandular epithelial cells for each of these antibody stains, all the epithelial cells were scored in representative sections from the mid-point of the uterus and means ± S.D. from a minimum of 6 mice were derived.

Biochemical analysis of cell cycle regulatory proteins

Uterine epithelial cell lysates were prepared in either the gel electrophoresis buffer or in kinase buffer, as described in detail before (Tong & Pollard 1999), at various times after hormone treatment using a method that gave an epithelial extract of <95% purity (Fagg *et al.* 1979, Tong & Pollard 1999). Protein concentration was measured using the Bradford reagent. Equal amounts of protein per treatment group were separated by SDS–gel electrophoresis and transferred to Immobilon P membrane (Millepore, Billerica, MA, USA). After blocking non-specific binding using dried milk, filters were probed with antibodies against cyclin D1, E and A, pRb and p107, and CDK4, CDK2 and PCNA as described (Tong & Pollard

1999). Specific binding was detected using an enhanced chemoluminescence system after incubation with an appropriate secondary antibody linked to horseradish peroxidase as described (Tong & Pollard 1999). The immunodetection for each protein was shown to be in the linear portion of the curve by titration of the lysates and antibody, and the signal was quantified by densitometry (Tong & Pollard 1999).

Immune complex kinase assays were performed after immunoprecipitation of the uterine lysates with anti-cyclin E, anti-cyclin A, anti-CDK4 and anti-CDK2 antibodies as described (Tong & Pollard 1999, Chen & Pollard 2003). Histone H1 was used as the substrate for anti-cyclin E, cyclin A and CDK2 immunoprecipitates while GSK pRb (Amino acids 769–921, Santa Cruz) was used as the substrate for CDK4. The reaction products were separated by SDS–gel electrophoresis followed by autoradiography as described (Tong & Pollard 1999). All experiments involving protein analysis were derived from five mice per group and were repeated at least twice with similar results. Blots shown are representative of these repeat experiments.

Statistical analysis

Statistical analysis was performed using ANOVA with Dunnett's multiple comparisons test to determine the significance of the effect of tamoxifen at various times/doses versus the control. For single time point comparisons between E₂ and tamoxifen treatments, Student's *t*-test was used.

Results

Dose–response to tamoxifen

A single injection of 50 ng E₂ to ovariectomized mice stimulates uterine luminal and glandular cell proliferation with a peak of cells detected with anti-BrdU antibody in S-phase 15 h after administration. At this stage, ~90% of cells have incorporated BrdU. This dose of E₂ has been demonstrated to be maximal (Martin *et al.* 1973). Consistent with this induction of DNA synthesis there is a concurrent nuclear localization of PCNA, the δ-sub-unit of DNA polymerase, and cyclin A synthesis and its nuclear localization (Tong & Pollard 1999). E₂ also stimulates uterine edema and this is often used as a surrogate marker for estrogenicity (Martin *et al.* 1973). Consequently, we used the four parameters of BrdU incorporation, PCNA nuclear localization, cyclin A synthesis and nuclear localization, and uterine edema to determine the responsiveness of the mouse uterus to tamoxifen over a range of doses from 0.125 to 1.5 mg/mouse. These responses were measured at 16 h after hormone administration because this is also the time point of maximal response (see below).

Figure 1 Dose-responses for tamoxifen in the uterus. Ovariectomized mice were given tamoxifen subcutaneously over a range of doses from 0 to 1.5 mg/mouse as shown. Sixteen hours after treatment mice were killed and the following parameters determined as described in the Materials and Methods section. (A) Wet weight; (B–D) luminal epithelial cells positive for BrdU (B), cyclin A nuclear (C) and PCNA nuclear (D) staining. The insert in (C) shows epithelial cell cyclin A protein concentration at each dose as determined by Western blotting of equal concentrations of total epithelial cell lysates. E₂, 50 ng E₂; C, untreated control; T, tamoxifen (dose, mg/mouse). Results are means $\pm$ S.D. * $P < 0.01$, significantly different from the control. The effect of tamoxifen was highly significant for all parameters ($P < 0.001$).

Sixteen hours post-administration tamoxifen induced a doubling of uterine wet weight even at the lowest dose of 0.125 mg/mouse, with little further increase up to 1.5 mg/mouse (Fig. 1A; $P < 0.001$). This indicates a nearly maximal response at 0.125 mg/mouse. In contrast, a tamoxifen dose of 0.125 mg/mouse did not induce an increase in cells incorporating BrdU (Fig. 1B). This required 0.25 mg/mouse to produce a statistically significant increase ($P < 0.01$), with the plateau of response

attained at 0.5 mg/mouse when approximately 50% of cells were in DNA synthesis (Fig. 1B). This was significantly lower than the response to E₂ (data not shown; $P < 0.001$). Consistent with this increase in DNA synthesis, there was a dose-related increase in the concentration of cyclin A measured by Western blotting with anti-cyclin A antibodies in uterine luminal epithelial lysates (Fig. 1C, insert), although maximal amounts were not detected until 1.0 mg/mouse was administered, at which point it was

Figure 2 Time course of responses to tamoxifen in the uterus. Ovariectomized mice were treated with 1.0 mg/mouse tamoxifen and killed at the times shown on the X-axis (A–C). (A) Uterine wet weight; (B) % of cells positive for BrdU staining in the luminal (○) and glandular (●) epithelium; (♦) BrdU-positive cells in mice given 50 ng E₂ subcutaneously at time zero. (C) PCNA nuclear-positive luminal epithelial cells. In A–C results are shown as means ± s.d. **P* < 0.01, significantly different from control untreated mice. The effect of tamoxifen was highly significant in all groups (*P* < 0.001). (D) Immunostaining showing BrdU-positive cells in transverse sections of control, tamoxifen (T) and E₂ (E) -treated mice killed 16 h after administration. BrdU-positive cells are shown by the reddish-brown immunostain.

induced to a similar level to that attained after treatment with 50 ng E_2 . Cyclin A acts in concert with its CDK2 partner in the nucleus to phosphorylate nuclear localized pRb-family substrates required for progression through S-phase and it is therefore an index of DNA synthesis (Sherr 1996). Thus the percentage of cyclin A nuclear positive cells was scored following immunohistochemistry of transverse uterine sections using anti-cyclin A antibodies. The lowest dose of 0.125 mg did not cause a significant elevation in cyclin A positive cells. This required 0.25 mg ($P < 0.01$, control vs tamoxifen treated) with the dose-response reaching a plateau at 0.5 mg/mouse, exactly mimicking the dose-response of incorporation of BrdU, although with only 30% of the epithelial cells being scored positive for this cyclin at this time-point (Fig. 1C).

The nuclear localization of PCNA is also considered to be a marker of cell proliferation (Hyde-Dunn & Jones 1997). Tamoxifen significantly ($P < 0.001$) increased the percentage of cells with nuclear-associated PCNA and this reached a maximum of 80% positive cells at a dose of 0.5 mg/mouse (Fig. 1D). However, just as for uterine edema, there was also a significant response compared with untreated mice ($P < 0.01$) at 0.125 mg/mouse with ~30% of the cells being positive for nuclear PCNA (Fig. 1D).

In these preliminary experiments we have shown that tamoxifen induces a significant dose-dependent increase in DNA synthesis in the luminal epithelium of ovariectomized mice. Given that 0.5 mg/mouse marked the inflection point of the dose-response curve, 1.0 mg/mouse was chosen for subsequent experiments because this produced a maximal response for all four parameters measured. Since 50 ng/mouse E_2 is a maximal dose, this indicates that tamoxifen is ~200 000 times less potent than E_2 in this organ and never achieves full estrogenicity.

Time course of uterine response to tamoxifen

We next determined the time-course of responses to 1.0 mg/mouse tamoxifen in ovariectomized, primed mice. This dose induced a dramatic and significant ($P < 0.001$) increase in wet weight with a 50% increase within 4 h of administration ($P < 0.01$, control vs 4 h tamoxifen treated). This progressively increased over time to reach approximately threefold the control weight at 24 h where it remained throughout the duration of the experiment until 72 h post-injection (Fig. 2A). The pattern of BrdU incorporation into luminal epithelium cells followed that observed following a single injection of E_2 (Martin *et al.* 1973) with the first significant increase in BrdU-positive luminal epithelial cells found at 8 h after injection (Fig. 2B; $P < 0.01$). This value reached a peak at 12–16 h, followed by a steady decline to below control steady-state values by 48 h (Fig. 2B). The peak value of ~50% of luminal epithelial cells incorporating BrdU was significantly less than the ~90% observed following a single injection of

50 ng E_2 (Fig. 2B,D; $P < 0.001$ Student's *t*-test). This effect on BrdU incorporation is illustrated in Fig. 2D. BrdU-positive cells in the glandular epithelium followed a similar pattern except the amplitude was much lower both in the untreated control and in the drug-treated group (Fig. 2B). Furthermore, the incorporation of BrdU into these cells persisted over the 72 h of the experiment.

Nuclear association of PCNA in luminal epithelial cells was detected earlier and persisted longer than the incorporation of BrdU. Thus there was a significant increase at 4 h after treatment ($P < 0.01$). However, the plateau of PCNA-positive cells at 12 h after injection was achieved at the same time as the number of BrdU-positive cells but thereafter this persisted until 24 h as compared with the 16 h peak for BrdU incorporation, before declining to untreated ovariectomized control levels at 72 h (Fig. 2C). This shows that PCNA persists in the nucleus even when cells have ceased DNA synthesis.

These data confirm and significantly extend previous studies (Martin & Middleton 1978) that conclude that the proliferative responses to tamoxifen in uterine epithelial cells show a similar time course to those observed after E_2 treatment.

Tamoxifen regulation of the cell cycle machinery

The hyperphosphorylation of the pRb family of proteins is thought to be central to the regulation of the mammalian cell cycle (Sherr 1996). Consequently, we examined the phosphorylation of pRb and p107 in response to 1.0 mg tamoxifen for up to 48 h post-treatment. In the control untreated uterine epithelial lysates, both pRb and p107 mostly run as a single band on SDS-PAGE corresponding to the hypo-phosphorylated forms of these molecules (Fig. 3) (Tong & Pollard 1999). Eight hours after tamoxifen treatment phosphorylated bands characterized by a lower electrophoretic mobility were visible for both pRb and p107. These phosphorylated forms increased and persisted until 24 h after treatment before they declined 48 h post treatment (Fig. 3).

Phosphorylation of these pocket proteins is by the sequential activity of cyclin D1/CDK4 and 6, cyclin E/CDK2 and cyclin A/CDK2 (Roberts 1999). The luminal epithelial response to E_2 also follows this pattern of sequential kinase activation with the exception that cyclin D/CDK4 protein and activity is only slightly affected by E_2 . Instead, cyclin D1 accumulates in the nucleus where it gains access to its substrates (Tong & Pollard 1999). Thus, we measured cyclin D1-, E- and A-associated kinase activities and the cellular localization of cyclin D and A in the uterine luminal epithelium following tamoxifen treatment of ovariectomized mice. As had been determined for E_2 treatment, the luminal epithelial concentration of cyclin D1 showed only modest changes following tamoxifen treatment, with an initial small decline at 4 h followed by a doubling between 16 and 24 h after administration

Figure 3 Tamoxifen induces phosphorylation of the Rb family of proteins in the uterine luminal epithelium. Western blots of SDS-PAGE gels upon which were separated equal protein concentrations of uterine epithelial protein extracts isolated from mice at the times indicated following tamoxifen treatment. Blots were probed with anti-pRb (upper panel) and anti-p107 (lower panel) antibodies. In each case the slower migrating band represents the hyper-phosphorylated form (ppRb and pp107) of the protein. C, control untreated ovariectomized mice.

(Fig. 4A). This is similar to that described following E_2 treatment (Tong & Pollard 1999). CDK4 is an abundant protein whose cellular concentration remains constant and unaffected by E_2 (Tong & Pollard 1999). This, therefore, has been used as a loading control for these experiments and per mg of protein loaded onto the gel, CDK4 remained constant over the 72 h treatment period studied (Fig. 4A).

Consistent with these relatively small changes in cyclin D1 protein over the time course studied, the total CDK4 activity in epithelial lysates was relatively unchanged over the first 12 h after administration as assessed by an immune-complex kinase assay using pRb as a substrate (Fig. 4A). This activity declined modestly 16 to 24 h after tamoxifen treatment (Fig. 4A). However, immunohistochemical studies of the localization of cyclin D1 in luminal epithelium using an anti-cyclin D1 antibody indicated a marked nuclear accumulation ($P < 0.01$) at 8 h after treatment (Fig. 4B,C). This nuclear accumulation continued to reach a peak at 16 h when ~50% of cells were positive, before declining at 24 h to return to control levels by 48 h after treatment (Fig. 4B,C). Thus, as described for E_2 action (Tong & Pollard 1999) it is the nuclear localization of cyclin D1 that brings the CDK4 activity to the nucleus, thereby allowing phosphorylation of the pRb family of proteins and initiation of the uterine epithelial cell cycle.

After the initial phosphorylation of pRb and p107 by cyclin D1/CDK4 or CDK6, the subsequent phosphorylation is performed by CDK2 in association with cyclin E or cyclin A. As assessed by Western blotting, tamoxifen

produced a modest elevation in cyclin E concentrations in the luminal epithelial cells approximately 8 h after administration and this declined between 24 and 48 h to below the control untreated levels (Fig. 5A). In contrast and consistent with the documented sequence of events following E_2 treatment of the uterus (Tong & Pollard 1999), cyclin A concentration in the uterine epithelial cells did not show a change until 12 h after tamoxifen administration after which it reached a peak at 16–24 h before declining at 48 h (Fig. 5A). This was paralleled by the detection, by immunostaining, of nuclear cyclin A at 12 and 16 h post-tamoxifen treatment with an anti-cyclin A antibody (Fig. 5B).

CDK2 levels showed relatively minor changes in concentration following tamoxifen treatment of ovariectomized mice (Fig. 5A). However, tamoxifen produced a marked increase in the active form of CDK2 (Thr160p; Fig. 5A) that is detected as a band with greater electrophoretic mobility than the inactive form. This increased mobility is due to the removal of two inhibitory phosphorylations and the addition of an activating phosphorylation at Thr160, resulting in a total reduction in charge of one phosphate group (Fig. 5A). This active form begins to increase 4 h after tamoxifen treatment and reaches its maximum between 16 and 24 h post-tamoxifen treatment. Consistent with the appearance of this activated form of CDK2, the activity of cyclin E, cyclin A and total CDK2 kinase activity in the epithelial cells, as measured by immune-complex kinase assays, progressively increased following tamoxifen treatment of ovariectomized mice (Fig. 5C). The activity of cyclin E-associated kinase reached a maximum at 16 h before rapidly declining (Fig. 5C) while cyclin A-associated activity increased to its maximal level 12–16 h post-treatment and this was sustained until 24 h (Fig. 5C). This is consistent with the induction of cyclin A protein (Fig. 5A). The peak of total CDK2 activity was detected at 16 h after treatment and this is at the time of maximal active form detected on the Western blots (Fig. 5C) and co-incident with the peak in BrdU incorporation (Fig. 2B). The persistence of this total CDK2 activity through 24 h (Fig. 5C) is due to the cyclin A-associated activity. This is consistent with the role of CDK2 in promoting the G1 to S-phase transition and of its requirement in association with cyclin A for the transit of S-phase.

Discussion

Estrogens play an essential role in the development and function of the female reproductive tract. In adult mice at estrus and in humans during the proliferative phase, E_2 is the primary mitogen to the uterine epithelial cells acting through its receptor transcription factor, ER α (Tong & Pollard 2002). Estrogens are also mitogenic in the mammary epithelium that is also a target organ because of

Figure 4 Tamoxifen induces cyclin D1 translocation from the cytoplasm to the nucleus. (A) Equal protein concentrations of lysates of uterine epithelial cells isolated from ovariectomized mice treated with tamoxifen at the times shown were analyzed by Western blotting for cyclin D1 (top) and CDK4 (middle) concentrations. The other portion of the lysates was used for measurement of CDK4 kinase activity (bottom) by an anti-CDK4 immune-complex kinase assay using GST-Rb as a substrate. NRS, normal rabbit serum control for non-specific kinase activity. (B) Transverse sections of uteri isolated at 8 and 16 h after tamoxifen (Tam) treatment and immunostained with anti-cyclin D1 antibody. Control, untreated ovariectomized control. Arrows indicate some nuclear-positive cells. (C) Percentage of luminal epithelial nuclei stained positive for cyclin D1 at different times after tamoxifen treatment. Results are means $\pm$ s.d. * $P < 0.01$, significantly different from control untreated mice. The effect of tamoxifen was highly significant ($P < 0.001$).

Figure 5 Tamoxifen increases cyclin E- and A-associated CDK2 activity. (A) Western blots of equal protein concentrations of uterine epithelial cell lysates isolated at the times indicated, probed with anti-cyclin E, anti-cyclin A and anti-CDK2 antibodies as indicated. The active form of CDK2 runs as a band with a higher electrophoretic mobility (Thr160-p) than the inactive form (Thr 160). (B) Immunohistochemical localization of cyclin A on transverse sections of mouse uteri treated with tamoxifen (Tam) for the times shown. Arrows indicate some positive cells. (C) Immune-complex CDK2-kinase assays using histone H1 as a substrate of the other half of the lysates shown in A to measure cyclin E (top) and cyclin A (middle) -associated CDK2 activity and total CDK2 activity (bottom) at various times after tamoxifen treatment. C, ovariectomized untreated control.

expression of the ER (Bocchinfuso & Korach 1977, Daniel *et al.* 1987, Haslam 1988). In both the uterine and mammary epithelium, E_2 activates the cyclin/cyclin-dependent kinase cell cycle machinery. First, members of the cyclin D family, together with their partners CDK4 and CDK6, are activated followed by induction of cyclin E- and cyclin-A-CDK2 activity. This results in the sequential phosphorylation of the central cell cycle regulatory molecule, pRb, and progression into S-phase

(Clarke & Sutherland 1990, Tong & Pollard 2002, Doisneau-Sixou *et al.* 2003). In the uterus, in contrast to mammary epithelial cells in culture, E_2 does not result in an elevation of cyclin D1 early in the cell cycle but instead mobilizes a pre-existent cytoplasmic pool to move into the nucleus. Similarly, cyclin D1's cytoplasmic partner, CDK4, is also accumulated in the nucleus in response to E_2 and this complex results in the first pRb phosphorylation at Thr 807/811 specifically in these epithelial cells

(Tong & Pollard 1999, Chen & Pollard 2003). This appears to be a central node in the regulation of uterine epithelial cell proliferation by sex steroid hormones because progesterone, a hormone that completely inhibits the E_2 -induced uterine epithelial cell proliferation, blocks entry of cyclin D1 into the nucleus and completely inhibits pRb phosphorylation (Tong & Pollard 1999). The importance of the mobilization of cyclin D in the E_2 -induced proliferative response is further emphasized by studies of cyclin D1 null mutant mice that undergo E_2 -induced proliferation through compensation by cyclin D2 that completely replaces cyclin D1 in its nuclear accumulation, CDK4 activation and phosphorylation of pRb (Chen & Pollard 2003).

In both mice and humans, tamoxifen is an agonist in the uterus and an antagonist in the breast despite binding to the ER in both these tissues (Jordan & Morrow 1999). The ER has two transcriptional activation domains, AF-1 and AF-2. AF-1 activity is stimulated by tamoxifen binding but AF-2 is inhibited (Smith *et al.* 1997, McDonnell 1999) while E_2 activates both AF-1 and AF-2 (Webb *et al.* 1995, Smith *et al.* 1997, Jordan & Morrow 1999). This results in the differential recruitment of receptor co-factors that could result in interpretation of estrogen signals as either inhibitory or stimulatory (Smith *et al.* 1997, Jordan & Morrow 1999, Dutertre & Smith 2000, Pearce *et al.* 2003). Tamoxifen has also been reported to have some non-receptor-mediated effects (Altan *et al.* 1999). Thus, it is an important question whether its proliferative effects in the uterus use the same mechanism as E_2 or whether there is activation of alternative pathways. For example, there are reports that tamoxifen can activate cyclin E/CDK2 and thereby activate cell proliferation independently of cyclinD1/CDK4 (Dhillon & Mudryj 2002).

In this report we show that tamoxifen induces luminal and glandular epithelial cell proliferation with a time course similar to E_2 . In these responses it acts as a classical impeded estrogen with about 200 000-fold lower potency than E_2 and never achieves a full estrogenic response. However, unlike other impeded estrogens such as estriol (Martin *et al.* 1976), its effects persist particularly upon the glandular epithelium and edema, suggesting long-term low level receptor occupancy. This results in cystic hyperplasia upon continuous administration (Martin & Middleton 1978). The different estrogenic effects are also obviously differentially regulated by tamoxifen since a full response upon water imbibition is affected at doses that do not induce DNA synthesis, and these responses are greater and more persistent than observed with E_2 . This may suggest that as well as the proliferation regulation through the ER, there is some non-genomic action perhaps through the activation of VEGF (Hyder *et al.* 1996), known to be at least partially responsible for the edema in the uterus. It can also be concluded, as it has been by other workers (Carthew *et al.* 1999), that the uterotrophic effects of an estrogen as measured by increased wet weight is not

a reliable marker for the proliferative activity of these compounds. Interestingly, nuclear staining for PCNA, the δ sub-unit of DNA polymerase- α , is also increased significantly over control levels at tamoxifen doses that do not affect DNA synthesis. Furthermore, the expression of PCNA in response to maximal doses of tamoxifen continues beyond the time when the cells are actively synthesizing DNA. PCNA expression and nuclear localization is often used as a surrogate marker for cell proliferation (Hyde-Dunn & Jones 1997) but at least in the uterus it appears to be an unreliable marker for such events. Indeed, in the uterus it has been detected in apoptotic cells in the uterine lumen (Lai *et al.* 2000).

Despite these discrepancies in dose-response for some uterotrophic activities, the action of tamoxifen upon the cell cycle machinery in the uterine epithelium is similar to that observed upon E_2 stimulation. This cell proliferation as found after E_2 treatment is restricted to the luminal and glandular epithelium over the first 48 h of treatment, with no involvement of stromal cells. Tamoxifen causes the mobilization of cyclin D1 into the nucleus from the cytoplasm of these epithelial cells. This results in phosphorylation of the nuclear substrate, pRb and p107. Thereafter, cyclin E/CDK2 is activated, followed by a dramatic induction of cyclin A, and a further elevation of CDK2 activity. This results in cells entering into S-phase co-incident with the nuclear localization of PCNA. The kinetics of these effects are similar to those seen in mice treated with E_2 although the percentage of cells entering S-phase is lower. Thus tamoxifen appears to regulate the uterine epithelial cell cycle through the same mechanism as E_2 and does not do so by triggering a down-stream event such as cyclin E expression independent of the canonical activation of cyclin D1/CDK4. Since the ER is essential for the E_2 -induced uterine proliferative responses (Korach 1994), these data suggest that activation of the AF-1 transcriptional regulatory sequences on the ER is sufficient to induce uterine epithelial cell proliferation. It will be interesting to study what aspect of ER activation is required for the E_2 -induced proliferation response in the mammary epithelium since tamoxifen inhibits this activity. Studies in cell culture have shown that this inhibitory effect is usually by suppression of cyclin D1 synthesis and induction of the CDIs, p18^{INK4C}, p21^{WAF1} and p27^{KIP1} (Planas-Silva & Weinberg 1997, Doisneau-Sixou *et al.* 2003). In the uterus, these inhibitors do not appear to play essential roles in regulating cell proliferation since null mutations in them do not impair female fertility that is dependent upon this proliferation (Tong *et al.* 1998, Zindy *et al.* 2001). Thus the biological responses to tamoxifen in the uterus and mammary gland cells are opposite and therefore the definition of the molecular basis for them should help define the action of tamoxifen and E_2 in different target tissues and provide a rationale for more selective therapies against estrogen-responsive breast cancers.

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