Tamoxifen induces endometrial and vaginal cancer in rats in the absence of endometrial hyperplasia

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Tamoxifen was administered orally to neonatal rats on days 2–5 after birth and the subsequent effects on the uterus were characterized, morphometrically, over the following 12 months. Tamoxifen inhibited development of the uterus and glands in the endometrium, indicating a classical oestrogen antagonist action. Between 24 and 35 months after tamoxifen treatment there was a significant increase in the incidence (26%) of uterine adenocarcinomas and a 9% incidence of squamous cell carcinomas of the vagina/cervix in the absence of any oestrogen agonist effect in the uterus. This demonstrates that an oestrogen agonist effect is not an absolute requirement for the carcinogenic effect of tamoxifen in the reproductive tract of the rat. The unopposed oestrogen agonist effect of tamoxifen on the endometrium may not be the only factor involved in the development of endometrial cancers. It is possible that tamoxifen causes these tumours via a genotoxic mechanism similar to that seen in rat liver. However, using 32P-post-labelling we failed to find evidence of tamoxifen-induced DNA adducts in the uterus. Tamoxifen may affect hormonal imprinting of oestrogen receptor responses in stem cells of the uterus, causing reproductive tract cancers to arise at a later time, in the same way as has been proposed for diethylstilbestrol. If these rodent data extrapolate to women, then women who are taking tamoxifen as a chemopreventative may have an increased risk of vaginal/cervical cancer, as well as endometrial cancer.

Introduction

Much controversy has surrounded the finding of an increased risk of endometrial cancer in post-menopausal women taking tamoxifen as an adjuvant therapy for breast cancer (1–4). The current explanation for this is that tamoxifen acts as an agonist of oestrogen action in the uterus, leading to hyperplasia of the endometrium, and thus promotes endogenous initiated cells to neoplasias, as with hormone replacement therapy (5). This idea has been reinforced by numerous studies that have demonstrated thickening of the endometrium by ultrasonography during tamoxifen treatment, prior to the finding of polyps and adenocarcinomas of the endometrium (6). However, some endometrial tumours have been found to arise on a background of endometrial atrophy, which would not fit this theory (7,8). Endometrial adenocarcinomas associated with prolonged tamoxifen treatment have been attributed to a possible genotoxic effect of tamoxifen in the endometrium (9,10), although the evidence for this is not considered convincing (11), because of the low level of DNA adducts that have been found in endometrial biopsies from women taking tamoxifen (9,10). The concept that tamoxifen could be genotoxic to women derives from the accumulation of DNA adducts in the livers of rats given tamoxifen that precedes the formation of liver cancers in as little as a year (12–14).

Tamoxifen is currently under evaluation as a chemopreventative for breast cancer in women with a high familial risk for developing this disease. A dramatic chemopreventative effect has already been reported in one trial (15), although this has not yet been confirmed in two other smaller trials taking place (16,17). As the chemopreventative treatment period is likely to be longer than the treatment period for tamoxifen use in adjuvant therapy for breast cancer, the long-term safety of tamoxifen use becomes even more important for risk–benefit analysis of its chemopreventative use. It is thus important to determine the underlying mechanism(s) of tamoxifen’s uterine carcinogenicity in women.

To examine the role of an oestrogen agonist action of tamoxifen in the genesis of endometrial cancer and whether this is an essential requirement, tamoxifen was administered orally to neonatal rats on days 2–5 after birth at a dose of 1 mg/kg body wt/day. In the adult rat, tamoxifen acts as an antagonist of oestrogen action with regard to the uterus and its development (18,19) and should, therefore, not give rise to uterine tumours by an oestrogen agonist promoting effect on the endometrium. The subsequent development of the uterus was then monitored at three monthly intervals for the first year of life to determine whether such exposure affected normal development. Because naturally occurring uterine cancer is a late onset tumour in rats, the animals were maintained for a further 23 months to monitor the development of such tumours.

Materials and methods

Animals and treatment

Seventy-eight female neonatal Wistar (Han) rats were given tamoxifen (1 mg/kg body wt/day) orally in peanut oil/lecithin/condensed milk mixture (2:0.2:3 w/w) on days 2–5 after birth. A group of 72 control rats was similarly dosed with the peanut oil/lecithin/condensed milk mixture. At 3, 6, 9, and 12 months after treatment, groups of six control and treated rats were killed by pentobarbitone overdose and the uteri were removed, weighed and fixed in 10% neutral buffered formalin (NBF) for paraffin wax section preparation and morphometric histological evaluation. The remaining (54 and 48 treated and control, respectively) animals were maintained on SDS RM 1 diet with ad libitum access to food and water for their normal lifespan (up to 35 months) to detect any long-term effects on the reproductive tract of neonatal exposure to tamoxifen. This study was carried out under the authority of the United Kingdom Home Office, Animals (Scientific Procedures) Act 1986.

Morphometry and tumour classification

Morphometric examination of the number of uterine gland profiles in three representative transverse sections was carried out on periodic acid–Schiff base stained 5 μm sections, scoring only those gland profiles that were surrounded continuously by basement membrane as separate gland profiles. Tumours were

Abbreviations: DES, diethylstilbestrol; ER, oestrogen receptor; NBF, 10% neutral buffered formalin.
Immunostaining for nERα cavity or beyond. glandular endometrium and were invasive of the myometrium, the peritoneal cavity. Some produced metastases to the lung. One adenocarcinoma of the uterus was also found in one animal in the tamoxifen treatment group (Table I). There was a reduction in the expression of nERα in tumour cells according to the degree of differentiation into endometrial adenocarcinomas. Well-differentiated adenocarcinomas of the uterus expressed nERα strongly (Figure 3c), while loss of differentiation was associated with a loss or absence of nERα staining (Figure 3d). This was very similar to the progressive loss of nERα staining associated with the development of tamoxifen-induced altered liver foci expressing GST-P as they progressed to adenomas and carcinomas in the liver (20).

A further unexpected finding was the presence of a low, but significant, incidence of squamous cell carcinomas of the vagina/cervix (9%) associated with neonatal tamoxifen treatment (Table I) 24–29 months after treatment (Figure 4). None were found in the control animals. The squamous parts of these tumours were not positive for nERα expression.

\[3^{2}P\)-post-labelling for the detection of DNA adducts

Following \[3^{2}P\)-post-labelling of uterine DNA samples and analysis by HPLC with radiochemical detection or TLC (23), no DNA adduct peaks or spots could be detected in either the control or treated samples. The limit of detection of DNA adducts using these conditions has been established as 1 adduct/10\(^{9}\) nucleotides for HPLC and 0.5 adducts/10\(^{10}\) nucleotides for TLC (23). The detection limits are calculated assuming that the labelling efficiency is 100%.

**Discussion**

The significant increase in the incidence of adenocarcinomas of the endometrium could not be explained by an unopposed

### Table I. Tumour tabulation for the reproductive tract of rats orally dosed with tamoxifen (1 mg/kg/day) on neonatal days 2–5 (% incidence in parentheses)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumour total</th>
<th>Adenocarcinoma (uterus)</th>
<th>Adenosquamous carcinoma (uterus)</th>
<th>Squamous cell carcinoma (vagina/cervix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamoxifen</td>
<td>19/54 (35)(^{a})</td>
<td>13/54 (26)(^{b})</td>
<td>1/54</td>
<td>5/54 (9)(^{c})</td>
</tr>
<tr>
<td>Control</td>
<td>3/48 (6)</td>
<td>3/48 (6)</td>
<td>0/48</td>
<td>0/48</td>
</tr>
</tbody>
</table>

\(^{a}\)Fisher’s exact test, \(P = 0.0003\).

\(^{b}\)Fisher’s exact test, \(P = 0.01\).

\(^{c}\)Fisher’s exact test, \(P = 0.04\).

classified as adenocarcinomas of the uterus if they were derived from the glandular endometrium and were invasive of the myometrium, the peritoneal cavity or beyond.

**Immunostaining for nERα in tumours**

NBF fixed uterine sections (5 μm) were dewaxed with xylene, taken to water and microwaved in citrate buffer, pH 6.0, for 20 min at full power (700 W). Following microwaving, the sections were placed in distilled water at room temperature and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water. A mouse monoclonal anti-human oestrogen receptor (ER) antibody (NCR-ER-6F11) was obtained from Vector Laboratories and used at a dilution of 1 in 25 (20,21). NCR-ER-6F11 recognizes full-length nERα; it does not recognize full-length ERβ by western blot comparison (Vector Laboratories, personal communication). A positive control of human endometrium was included in all batches of immunostains. Immunoreactive nuclei were visualized using 3,3'-diaminobenzidine/H\(_{2}\)O\(_{2}\). Sections were lightly counterstained with haematoxylin.

**Detection of tamoxifen-induced \[3^{2}P\)-post-labelled DNA adducts**

Uterine DNA from rats treated with tamoxifen and their respective controls, killed 24 h after the last treatment (day 6), were analysed for tamoxifen DNA adducts by \[3^{2}P\)-post-labelling. DNA was extracted from the uteri of pooled litters (20 tamoxifen-treated and 20 control animals) by the method of Gupta (22) using proteinase K digestion, phenol/chloroform extraction and digestion with RNase A and RNase T1. DNA purity and concentration were established spectrophotometrically. Only DNA with an A\(_{260}\)/A\(_{280}\) ratio of 1.7–1.9 was used. \[3^{2}P\)-post-labelling was performed on 10 μg of each sample using nuclease P1 enhancement as previously described (14). \[3^{2}P\)-post-labelled DNA samples were then analysed by HPLC with on-line radiochemical detection or TLC (23).

**Statistical methods**

Differences in body and uterine weights were analysed by ANOVAR with Dunnett’s test for significance at the 5% level. Tumour incidences were compared using Fisher’s exact test.

**Results**

**Tamoxifen-related effects on body and uterine weights**

Body weights at 3, 6, 9 and 12 months were not significantly affected by treatment (data not shown) while uterine weights (expressed as a percentage of body weight) were significantly reduced at 3 months after treatment, but gradually recovered after this time (data not shown). The stage of the oestrous cycle was not determined for individual animals at death.

**Tamoxifen-related effects on endometrial gland genesis**

Figure 1 shows the inhibition of development of the uterine glands in the endometrium subsequent to neonatal tamoxifen treatment on days 2–5. This demonstrates that there was no chronic oestrogen agonist effect of tamoxifen; on the contrary, tamoxifen treatment at this developmentally sensitive time after birth inhibited the subsequent normal development of the endometrium and uterus.

**Tumours induced by neonatal exposure to tamoxifen**

Between 24 and 35 months following neonatal tamoxifen exposure, 26% of tamoxifen-treated animals had developed uterine endometrial adenocarcinomas (Figure 3a), compared with 6% in the control group (Figure 2 and Table I). These were locally invasive tumours, which penetrated into and through the myometrium (Figure 3b) and into the peritoneal cavity. Some produced metastases to the lung. One adenosquamous carcinoma of the uterus was also found in one animal in the tamoxifen treatment group (Table I). There was a reduction in the expression of nERα in tumour cells according to the degree of differentiation into endometrial adenocarcinomas. Well-differentiated adenocarcinomas of the uterus expressed nERα strongly (Figure 3c), while loss of differentiation was associated with a loss or absence of nERα staining (Figure 3d). This was very similar to the progressive loss of nERα staining associated with the development of tamoxifen-induced altered liver foci expressing GST-P as they progressed to adenomas and carcinomas in the liver (20).

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**Discussion**

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Tamoxifen-induced reproductive cancers in rats

Fig. 2. Cumulative mortality for tamoxifen-treated and control rats and the development of adenocarcinoma of the uterus.

Fig. 3. Pathology of reproductive tract tumours in tamoxifen-exposed rats. (a) Glandular part of uterine adenocarcinoma induced by tamoxifen. H&E, ×250. (b) Adenocarcinoma of the uterus invading the myometrial wall of the uterus. H&E, ×250. (c) nERα immunostaining of well-differentiated uterine adenocarcinoma. Note uniform staining of glandular nuclei. Immunoperoxidase, ×250. (d) nERα immunostaining of less well differentiated uterine adenocarcinoma. Note the loss of nERα staining in the poorly differentiated part of the tumour. Immunoperoxidase, ×250.

An oestrogen agonist effect of tamoxifen, as this had been shown not to occur (Figure 1). The present rodent study shows that tamoxifen can cause uterine cancers in rats in the absence of a sustained simple unopposed oestrogen agonist effect on the endometrium. However, it is still possible that tamoxifen acts as an oestrogen agonist only during the time of exposure on
Tamoxifen has been shown to induce ER expression in luminal epithelial cells of the neonatal mouse, at a time when this expression is not normally apparent (29), indicating a possible hormonal imprinting effect on ER expression and perhaps subsequent function.

Clearly, the great sensitivity of the neonatal rodent to the carcinogenic potential of endocrine-disrupting chemicals and drugs makes them ideal for studying this process, independently of concerns over whether or not the compounds are agonists or antagonists of oestrogen action, in the reproductive tract.

The present data indicate that tamoxifen is able to induce cancers of the uterus and vagina in the rat, even when there is no oestrogen agonist effect evident, which must give cause for concern over its use as a chemopreventative agent, whatever mechanism is responsible. Hormonal imprinting has been suggested to be a possible mechanistic factor in the increased incidence of a rare rete testis carcinoma found in mice treated in utero with DES (26) and may also be involved in the induction of mouse uterine adenocarcinomas by tamoxifen (28). A similar mechanism could contribute to the development of endometrial and vaginal cancers in rats.

Acknowledgements

The authors would like to thank Retha Newbold for her encouragement with this work, Jennifer Edwards and Linda Wilkinson for preparing the histology sections and Michael Festing for performing the statistical analyses.

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Received September 28, 1999; revised November 16, 1999; accepted November 25, 1999