The levonorgestrel-releasing intrauterine device induces endometrial decidualisation in women on tamoxifen

by

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Abstract

There is conflicting literature on whether the levonorgestrel-releasing intrauterine system (LNG-IUS; Mirena®) induces decidualisation in the tamoxifen-treated endometrium. The expression of the decidualisation marker IGFBP-1 was measured using immunohistochemistry in endometrial biopsies and in serum (using ELISA) of 20 postmenopausal women at the start of tamoxifen-treatment for breast cancer. Ten women were then fitted with LNG-IUS and the other ten received tamoxifen-treatment only and acted as controls. Samples were taken at baseline and after 12 months. At baseline, all endometrial samples were negative for IGFBP-1 and at 12 months, IGFBP-1 was only expressed in the endometria of women fitted with the LNG-IUS, confirming the observed histological features of decidualisation. By contrast, serum IGFBP-1 concentrations were increased by tamoxifen, but not in the group receiving LNG-IUS. In conclusion, tamoxifen induces a rise in serum IGFBP-1 suggesting a systemic, possibly hepatic effect, whilst LNG abrogates this in both the liver and endometrium.

Impact statement

What is already known on this subject?

Previous reports of the use of LNG-IUS in women on tamoxifen have provided conflicting evidence as to whether the endometrium exhibited decidualisation or not. These reports were however based solely on histological examination and lacked supporting biochemical data.

What do the results of this study add?

After 12 months of treatment with LNG-IUS, the endometria of women on tamoxifen show histological features of decidualisation and the presence of the decidualisation marker IGFBP-1, suggesting that levonorgestrel protects the tamoxifen-treated uterus from additional pathology by causing decidualisation. Serum levels of IGFBP-1 were expected to be a reflection of uterine production, but contrary to expectations, higher levels were identified in women on tamoxifen alone. These data suggest that an inhibition of tamoxifen-induced serum IGFBP-1 production (possibly from a hepatic source) by LNG-IUS occurred and indicates independent systemic effects of both drugs in post-menopausal breast cancer patients.

What are the implications of these findings for clinical practice and/or further research?

This research demonstrated a mechanism for endometrial protection in women on tamoxifen. It also alerts clinicians to the fact that both tamoxifen and LNG-IUS exert systemic effects in this patient group.

Introduction

Tamoxifen is a selective estrogen receptor modulator that acts as a competitive estrogen receptor antagonist and significantly improves overall survival in women with estrogen receptor positive breast cancer (Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2005). It exerts a proliferative, estrogen-like effect on the endometrium thus increasing the risk of endometrial polyps, hyperplasia and cancer. A recent Cochrane review concluded that the insertion of the levonorgestrel-releasing intra-uterine system (LNG-IUS) Mirena® in tamoxifen users led to a significant reduction in the incidence of endometrial polyps over 12 months (OR 0.22, 95% CI 0.08 to 0.64) and 24-60 months (OR 0.22, 95% CI 0.13 to 0.39). In addition, the LNG-IUS reduced the incidence of endometrial hyperplasia over long-term follow-up (OR 0.13, 95% CI 0.03 to 0.6), possibly through decidualisation (Dominick *et al.*, 2015).

Levonorgestrel is a synthetic 19-nortestosterone progestin derivative that is widely used in contraception and postmenopausal hormone therapy. The LNG-IUS releases 20 μg of levonorgestrel per 24 hours into the uterine cavity. The local effect predominates but maximum serum levonorgestrel concentrations are reached within a few hours following insertion, and are maintained at 150-200 pg per ml (Lahteenmaki *et al.*, 2000). The LNG-IUS, via the effects of locally delivered levonorgestrel, may prevent tamoxifen-induced polyps (Gardner *et al.*, 2009). Gardner *et al.* reported that all women in whom the LNG-IUS was fitted (n=40) showed histological evidence of a decidualised endometrium at the end of an average of 27.9 months of use. The authors linked the inhibition of polyp formation to the 'progestogenic antagonism' of tamoxifen effects (Gardner *et al.*, 2009). Chan *et al.* (2007) also reported a reduction in polyps in LNG-IUS users, but also that the endometrium at 12 months was predominantly atrophic (Chan *et al.*, 2007).

The aim of this study was to examine the effect of LNG-IUS on the expression of IGFBP-1 (as a marker of stromal cell decidualisation) in tamoxifen-treated postmenopausal women and its effect on their serum IGFBP-1 concentrations.

Materials and Methods

Ethics Statement

All clinical samples were collected from patients attending the University Hospitals of Leicester NHS Trust, Leicester, UK, according to guidelines set out by the Leicestershire, Northamptonshire & Rutland Local Research Ethics Committee (Approval number 07517). All volunteers provided written informed consent prior to enrolment and sample collection.

Sample collection and processing

Endometrial pipelle biopsies and serum samples were obtained from 20 postmenopausal women with breast cancer scheduled to receive adjuvant tamoxifen (20mg daily). None of the participants had received any exogenous steroids since the diagnosis of breast cancer and all had healthy pelvic organs confirmed by digital examination and transvaginal ultrasound. Biopsies (n=20) were obtained from women participating in a randomised controlled trial, where randomisation was achieved using a sealed envelope method Women were allocated to receive the the 52 mg LNG-IUS (Mirena®, Bayer Oy, Turku, Finland) (n=10), or to the control group (n=10) who received tamoxifen only. All women had endometrial pipelle biopsies and venous blood samples obtained at the start of treatment (denoted C-0 and LNG-0), and again after 12 months (denoted C-12 and LNG-12). In addition, first trimester trophoblast and serum obtained from women undergoing surgical termination of pregnancy and archival liver biopsy were used as positive controls for IGFBP-1 expression.

Venous blood (5ml) was collected from an antecubital vein using an aseptic technique into serum-separating vacutainers (Starstedt, Leicester, UK) and transported to the laboratory at room temperature. After a clot had formed and retracted, the blood was centrifuged at 1200g for 30 minutes and serum (2ml) stored at -20^{0}C for biochemical analysis.

IGFBP-1 ELISA

Serum IGFBP-1 concentrations were measured using the DSL-10-7800 ACTIVE® Total IGFBP-1 ELISA Kit according to the manufacturer's instructions (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). In addition to the 7 standards and 2 control samples with known IGFBP-1 concentrations supplied in the kit, 3 further serum samples obtained from women in the first trimester of pregnancy were used as controls. The minimum

detection limit for the assay is 0.25 ng/ml. The intra-assay and inter-assay coefficients of variation were 1.7–4.6% and 6.2–7.6%, respectively. Absorbance was read using a Multiskan Ascent ELISA plate reader (Labsystems Diagnostics, Vantaa, Finland), with the detection filter set at 450 nm and the reference set at 620 nm. Background absorbance was subtracted and the mean absorbance calculated from duplicate values. IGFBP-1 concentrations were calculated by interpolation from the standard curve.

Immunohistochemistry

Endometrial biopsy samples were fixed in 10% formal saline and processed into paraffin embedded blocks. Five micron sections were obtained from each block on silane-coated slides for immunohistochemistry and haematoxylin and eosin staining.

Immunohistochemistry was performed using a modification of the method of Sugita *et al*. (Sugita *et al.*, 2011). Briefly, antigen retrieval was by microwave in 10mM citric acid buffer (pH 6.0) for 20 minutes at 800 Watts. Endogenous peroxidase was suppressed by hydrogen peroxide and non-specific binding was blocked with 10% normal rabbit serum (Dako, Glostrup, Denmark). Avidin (Vector Laboratories, Peterborough, UK) and biotin blocking (Vector Laboratories) solutions were applied to the sections. Mouse anti-human IGFBP-1 (H-3, Santa Cruz Biotechnology, Insight Biotechnology, Wembley, Middlesex, UK) was applied to the slides at 1:125 dilution and then incubated at 4°C overnight. Mouse IgG (Vector Laboratories) was applied to the negative controls. Biotinylated rabbit anti-mouse IgG $(Fab')_2$ fragments (Dako) diluted to 1:400 in PBS was applied for 90 minutes at room temperature. ABC Elite and 3, 3'-diaminobenzidine (DAB) substrate (Vector Laboratories) were used as detection systems and slides were lightly counterstained with Mayer's haematoxylin (Sigma), washed and mounted. Photomicrographs were obtained using a single chip colour video camera (Sony DXC-151P, Tokyo, Japan), and Axioplan microscope (Carl Zeiss, Milton Keynes, Herts. UK) and Axiovision software (version 4.0, Carl Zeiss, Munich, Germany). Images were taken at 200x magnification.

Data collection and statistical analysis

Power analysis for serum IGFBP-1 measurements with $\alpha = 0.05$ and $\beta = 0.80$ for a 50% difference indicated a minimum of five patients was required in each arm of the study. The mean and standard error of the serum IGFBP-1 concentrations of the four patient groups were

compared using paired Student's *t*-test using serum IGFBP-1 concentrations at baseline as the reference; $p<0.05$ was considered to be statistically significant. Data was analysed using Graphpad Prism (version 6.07).

Results

Tamoxifen-treatment and group identity

There were no significant differences between the groups with respect to age, BMI, or time since the menopause (Table 1). The mean duration of tamoxifen treatment prior to the first endometrial and blood sample was 45.6 days and was not significantly different between the control (47.1 ± 22.8 days; mean ± SD) and LNG-IUS group (44.0 ± 27.3 days; p=0.7). [Insert Table 1 here]

Histology and Immunohistochemistry

Biopsies from both LNG-0 and C-0 groups had comparable histological features: 3 in each were inadequate and 7 were inactive or atrophic (Figure 1A and Figure 1B). None exhibited features of decidualisation (Figure 1E and Figure 1F). C-12 samples were either inadequate (n=5) or inactive (n=5) (Figure 1C). Most (n=9) LNG-12 biopsies exhibited features of decidualisation, and one was inadequate (Figure 1D). Decidualised stromal cells featured plump, polygonal cells and abundant stromal leukocytic infiltrates and this was confirmed by immunohistological expression of IGFBP-1 in all the nine samples. [Insert Figure 1 Here]

Serum IGFBP-1

Figure 2 shows that there was no significant difference in the serum IGFBP-1 concentrations between C-0 (17.4 ± 3.6 ng/ml) and LNG-0 (22.3 ± 5.3 ng/ml; p=0.4). After 12 months, serum IGFBP-1 concentration increased in the C-12 group to 34.7 ± 9.3 ng/ml, but not increased in the LNG-12 (20.6 ± 2.4 ng/ml) group. The difference between C-12 and LNG-12 was statistically significant (p=0.03). [Insert Figure 2 here]

Discussion

Morphological changes in stromal fibroblasts and IGFBP-1 expression are markers of decidualisation. IGFBP-1 is one of a family of structurally related soluble proteins that modulates the bioavailability of insulin-like growth factor I (IGF-1) (Fowler *et al.*, 2000). Endometrial IGFBP-1 expression is found in the stroma of late secretory endometrium and IGFBP-1 mRNA and protein are highly expressed in the decidua during pregnancy, with levels peaking around 16 weeks of gestation (Wathen *et al.*, 1993).

Case reports published between 1989 and 1994 demonstrated stromal decidualisation in postmenopausal women receiving tamoxifen and megestrol acetate – a progestogen (Nuovo *et al.*, 1989, Nasri *et al.*, 1991, Cohen *et al.*, 1992, Corley *et al.*, 1992, Lazebnik *et al.*, 1994). Cohen *et al.* studied 12 asymptomatic postmenopausal women using tamoxifen and either megestrol acetate or medroxyprogesterone acetate. The endometrium exhibited a decidual reaction in all except one woman who received progestin therapy for less than one month (Cohen *et al.*, 1996). However, Powles *et al.* (1998) did not report decidualisation in their series of 39 postmenopausal women receiving tamoxifen and cyclical norethisterone for 21 out of 28 days for 3 months (Powles *et al.*, 1998). Decidualisation was also not reported in the study by Chan *et al*. (2007), but was reported in the study by Corley *et al*. (1992).

Our study confirmed decidualisation both histologically and by using IGFBP-1 expression after concurrent administration of tamoxifen and LNG in postmenopausal women over 12 months. This is in keeping with the findings of previous studies by our group (Gardner *et al.*, 2009). The design of the present study does not allow assessment of the time of onset of these changes but the findings are in agreement with features observed after one year of LNG-IUS use in premenopausal women (Pengdi *et al.*, 1999).

Serum levels of IGFBP-1 in women fitted with the LNG-IUS did not alter significantly after 12 months. It has previously been shown that the changes in endometrial IGFBP-1 expression during the normal menstrual cycle are not reflected in serum levels (Thierry van Dessel *et al.*, 1996). Pakarinen *et al*. (1999) found no change in serum IGFBP-1 levels in a cohort of pre-menopausal women after 3 months of treatment with LNG-IUS. These observations are in contrast to the changes observed during pregnancy, where the decidua is considered the primary source of the elevated IGFBP-1 in maternal serum (Ingec *et al.*, 2004), whereas in

non-pregnant women the primary source of IGFBP-1 is the liver (Leu and George, 2007). It has been argued that pseudo-decidual cells identified following progestogen administration are distinct from decidual cells identified in pregnancy (Bell, 1990), where decidual differentiation is associated with a dramatic increase in IGFBP-1 synthesis from existing gene transcripts rather than through nascent gene expression.

The increase in serum IGFBP-1 in the control group after 12 months of tamoxifen treatment, is in keeping with findings of other studies, where tamoxifen has been shown to supress plasma levels of IGF-1, most of which circulates bound to IGF binding proteins (Colletti *et al.*, 1989). Lonning *et al.* confirmed that in addition to its effect on IGF-1, tamoxifen increased plasma IGFBP-1 concentration by a mean value of 78% in postmenopausal patients with breast cancer (Lonning *et al.*, 1992). The observed difference in serum IGF-BP1 between the two groups can be explained by a systemic inhibitory effect of LNG.

Critchley *et al.* demonstrated that histological changes following insertion of the LNG-IUS are not limited to the contact site of the device but are manifest throughout the uterus (Critchley *et al.*, 1998a). Within 1 month of intrauterine levonorgestrel exposure, endometrial glands were reported to become largely atrophic (Silverberg *et al.*, 1986, Critchley *et al.*, 1998a, Critchley *et al.*, 1998b). Hejmadi *et al.* also reported predominantly atrophic endometrial glands in women fitted with LNG-IUS for between 4 and 70 months (Hejmadi *et al.*, 2007). This effect may be secondary to down regulation of steroid receptor expression following prolonged progestogen administration (Critchley *et al.*, 1998a). The finding of pre-decidual changes in the tamoxifen plus LNG treated endometrium may be mediated by increased expression of both estrogen and progesterone receptor isoforms in the postmenopausal endometrium (Leao *et al.*, 2013).

Emerging evidence points to differences between the clinical and biochemical effects of progesterone and progestogens when combined with estrogen in hormone replacement therapy and there is evidence that the increased breast cancer risk in HRT users is related to the progestogenic component (Carroll *et al.*, 2017). Thus, while there is no evidence that the use of LNG-IUS is detrimental to the risk of recurrence of breast cancer for women on tamoxifen, establishing the effect, if any, will require larger scale studies.

In conclusion, this study demonstrated decidualisation in the endometrium of tamoxifen-treated postmenopausal women 12 months following LNG-IUS insertion. Tamoxifen alone, but not when combined with LNG-IUS, induced an increase in serum IGFBP-1. Thus, serum IGFBP-1 levels are likely to be a reflection of systemic rather than local effects of tamoxifen and levonorgestrel.

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Figure Legends

Figure 1. IGFBP-1 expression in endometrial biopsies and control tissues.

IGFBP-1 immunostaining of a control group sample at baseline (C-0: **Panel A**) and after 12 months (C-12: **Panel B**) are shown with the LNG-IUS group at baseline (LNG-0; Panel C) and at 12 months (LNG-12; **Panel D**). A sample from first trimester decidua (**Panel E**) and liver biopsy (**Panel F**) are used as positive tissue controls.

Figure 2. Serum IGFBP-1 concentrations at study entry and after 12 months.

Serum IGFBP-1 in women in the control group at baseline (C-0) and after 12 months (C-12) and in the LNG-IUS group at baseline (LNG-0) and after 12 months (LNG-12). The data are presented as the mean ± SE (n=10 per group). *P < 0.05 compared to baseline (Student's paired *t*-test).

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