SHORT COMMUNICATION

Lack of evidence from HPLC $^{32}$P-post-labelling for tamoxifen–DNA adducts in the human endometrium

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Tamoxifen is associated with an increased incidence of endometrial cancer in women. It is also a potent carcinogen in rat liver and forms covalent DNA adducts in this tissue. A previous study exploring DNA adducts in human endometria, utilizing thin layer chromatography $^{32}$P-post-labelling, found no evidence for adducts in tamoxifen-treated women [Carmichael,P.L., Ugwumadu,A.H.N., Neven,P., Hewer,A.J., Poon,G.K. and Phillips,D.H. (1996) Cancer Res, 56, 1475–1479]. However, subsequent work utilizing HPLC $^{32}$P-post-labelling [Hemminki,K., Ranjaniemi,H., Lindahl,B. and Moberger,B. (1996) Cancer Res, 56, 4374–4377] suggested that very low levels could be detected. We have sought to investigate this question further by reproducing the HPLC methodology at two centres, and analysing endometrial DNA from 20 patients treated with 20 mg/day tamoxifen for between 22 and 65 months. Liver DNA isolated from tamoxifen-treated rats was used as a positive control. We found no convincing evidence for tamoxifen-derived DNA adducts in human endometrium. HPLC elution profiles of post-labelled DNA from tamoxifen-treated women were indistinguishable from those obtained with DNA from 14 untreated women and from six women taking toremifene, an analogue of tamoxifen.

Tamoxifen has been available since the early 1970s (1978 in the USA) as a first-line treatment of metastatic breast cancer in post-menopausal women. It is now the adjuvant hormonal therapy of choice for treatment of node-positive or node-negative disease, and is currently being assessed for prophylactic use, with mixed initial success (1–3). However, the association of the drug with human endometrial cancer has been the cause of considerable controversy. Several studies have suggested an association between the use of tamoxifen in breast carcinoma patients and the subsequent development of endometrial cancer (4–6). Indeed, in 1996 IARC concluded that tamoxifen is a human carcinogen, increasing the risk of endometrial carcinoma in breast cancer patients (7). Debate as to the mechanism(s) of this effect has centred on whether tamoxifen is genotoxic to human tissues, as is the case in the rat liver where it is metabolized to $\alpha$-hydroxytamoxifen, the proximate carcinogen, which is activated by further metabolism to a sulphate ester which gives rise to high levels of DNA adducts (8,9). In addition to forming DNA adducts, tamoxifen can also induce chromosomal changes in rat hepatocytes (10) and mutations in the tumour suppressor gene, p53 in rat liver tumours (11). These genotoxic events manifest as hepatocellular carcinoma in tamoxifen-treated rats (12,13). In humans, however, no evidence has emerged of any increase in liver cancer risk following tamoxifen treatment and, in contrast to the rat, DNA adducts do not appear to be formed by tamoxifen in the human liver (14) or primary cultures of hepatocytes (15). Hence, it is only in the uterus of humans that tamoxifen is carcinogenic, a tissue where the drug acts as a partial agonist rather than oestrogen antagonist (16).

A study exploring DNA adduct formation in human endometria, utilizing thin layer chromatography (TLC) $^{32}$P-post-labelling, found no evidence for such tamoxifen adducts in women treated with the drug (17). Furthermore, in this study it was demonstrated, using endometrial explant cultures, that the minimum concentration of $\alpha$-hydroxytamoxifen required to give detectable levels of DNA adducts in the cultures was 10 000 times greater than the concentration measured as a circulating metabolite in patients receiving tamoxifen treatment. However, a subsequent study utilizing high performance liquid chromatography (HPLC) $^{32}$P-post-labelling suggested that very low levels of adducts (2.7 adducts/$10^9$ bases) could be detected in five out of seven endometrial samples from six patients treated with 20 or 40 mg/day tamoxifen (18). A subsequent preliminary report has also claimed to have detected tamoxifen-associated adducts in human endometrium (19). Doubts have been raised (20) about the conclusions drawn from the original HPLC $^{32}$P-post-labelling study (18) in view of the marginal nature of the putative adduct peaks detected. Clearly, it is important to clarify these discrepancies and to elucidate the actual mechanism(s) of tamoxifen-associated endometrial carcinoma, particularly considering the current proposal to use tamoxifen prophylactically to prevent breast cancer in healthy women.

The current study was carried out in order to investigate further whether tamoxifen forms DNA adducts in human endometria. Reproducing the HPLC $^{32}$P-post-labelling methodology (18) in two laboratories and modifying it further, we have analysed endometrial DNA from 20 patients treated with 20 mg/day tamoxifen for periods ranging from 22 to 65 months (age range 56–73 years). In addition, we have compared the results obtained with tamoxifen-treated patients with endometrial DNA from six toremifene-treated patients (60 mg/day for 6 or 12 months) and from 14 drug therapy-free control patients. Comparison of analyses of all human samples was made with data obtained from liver DNA from tamoxifen-

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin layer chromatography.
Fig. 1. HPLC analyses of $^{32}$P-post-labelled DNA from: (A and B) tamoxifen-treated rat liver; (C–G) endometrial DNA from women treated with tamoxifen; (H) endometrial DNA from woman treated with toremifene; (I) endometrial DNA from control patient. (A), (C) and (D) were performed using HPLC system 1. (B) and (E–I) were performed using HPLC system 2. (G–I) were performed using enhanced digestion/labelling procedures (36 mU micrococcal nuclease and 300 ng spleen phosphodiesterase/µg DNA, 37°C, 17 h; 300 ng/µg nuclease P1; post-labelling with 25 µCi $[^{32}$P]$\text{ATP}$). Bars in (C–F) denote the position at which an adduct peak would have been expected, had it been present. Arrows in (G–I) indicate ‘adduct’ peaks at similar retention times to that of the major rat liver tamoxifen–DNA adduct in HPLC system 2.

treated rats [female Fischer F344 rats (120 g) administered tamoxifen in tricaprylin (100 µl/100 g) by gavage at a dose of 0.6 nmol/kg; the rats were killed 24 h after treatment].

Direct injection HPLC $^{32}$P-post-labelling was performed on two systems directly reproducing the micrococcal nuclease/spleen phosphodiesterase DNA-digestions, nuclease P1-digestions and $^{32}$P-labelling procedures of Hemminki et al. (18). System 1 consisted of twin Shimadzu LC6A pumps, a Waters Wisp auto-injector and Nuclear Enterprises Isoflo radioactivity detector with Ramona data capture. System 2 comprised a Waters 2690 Separations Module and Packard Flow Scintillation Analyser with Millennium data capture. Both systems utilized Phenomenex Kromosil C18, 250×2 mm, 5 µm columns, eluted with binary gradients of 0.2 M ammonium formate, pH 4.2 and methanol; 2% methanol for 5 min, linearly increased to 70% in 65 min, then linearly increased to 100% in 5 min, maintained at 100% for 10 min before being linearly decreased to 2% in 10 min; flow rate 0.25 ml/min; direct injection of 10 µg $^{32}$P-post-labelled DNA digest. Radioactivity detection was performed for each complete run on system 1, but on system 2, the flow was diverted from the detector for the first 16.7 min.

In the initial analyses, we compared HPLC elution profiles of the human DNA (examples in Figure 1C–F) with those of liver DNA from tamoxifen-treated rats (Figure 1A and B) and found no evidence for the presence of tamoxifen-derived DNA adducts in any of the human samples on either HPLC system. Furthermore, we found no evidence for endometrial DNA...
adducts induced by the tamoxifen analogue, toremifene (data not shown).

In subsequent analyses we altered the primary and secondary digestions of DNA samples, increasing the concentrations of micrococal nuclease, spleen phosphodiesterase and nuclease P1 (by 2.5 times each), and increased the [32P]ATP concentration (by 3.6 times). Under these conditions we increased the detection of the rat liver tamoxifen adduct standard by ~4-fold. Allowing for variations in background levels of radioactivity, a conservative estimate of the limit of detection of these procedures is 1 adduct/10⁹ nucleotides/10 µg DNA. Utilizing the improved protocol we re-analysed DNA from 14 control patients, 14 tamoxifen-treated patients and six toremifene-treated patients on system 2. With these conditions we found that we could detect radioactive peaks at or very close to the retention time of the tamoxifen adduct standard (Figure 1G–I). However, these peaks with very similar retention times to the standard were detected in five out of 14 control patients, seven out of 14 tamoxifen patients and three out of six toremifene patients.

Hence, we have demonstrated that radioactive peaks with the retention time of the major rat liver tamoxifen–DNA adduct are detectable, using HPLC 32P-post-labelling, in the DNA of tamoxifen-treated patients, but are also present in a similar proportion of control patients and toremifene-treated patients using the same assay system, and at similar levels of detection. Thus, these ‘adducts’ are probably endogenous species or artefacts detectable by the highly sensitive 32P-post-labelling procedure. Indeed, in subsequent experiments we have shown that this putative ‘adduct’ can be detected in untreated salmon sperm DNA and may be derived from the [γ-32P]ATP used in the technique (P.L. Carmichael et al., unpublished data).

When the DNA digests were subjected to preliminary chromatography on PEI-cellulose in 2.3 M phosphate, and material retained at the origin eluted with 4 M pyridinium formate and subjected to HPLC, radioactive peaks with the retention time of the rat liver tamoxifen–DNA adduct were not observed with any of the human endometrial DNA samples or with the salmon sperm DNA. In contrast, the efficiency of recovery of the rat liver adduct was 70% in this procedure.

It is interesting to note that in the study by Hemminki et al. (18) small peaks were also seen in control endometrial samples and, indeed, collection of the peak from a control patient, and re-analysis using two-dimensional TLC also gave a weak ‘adduct’ spot at a similar plate position as a tamoxifen-treated patient, albeit at a lower spot intensity.

On the basis of this evidence and our previous studies (16,21,22), it would appear that neither tamoxifen nor toremifene is metabolized in women to electrophiles that bind to DNA in endometrium in sufficient quantity to implicate a genotoxic mechanism for carcinogenicity. A significant difference between rats and humans appears to be that, whereas α-hydroxytamoxifen is activated by rat hydroxysteroid sulphato-transferase to mutagenic and DNA-binding products, it does not appear to be a good substrate for any of the known human sulphotransferases (23).

However, the possibility that tamoxifen and toremifene may have other effects on human endometrial tissue cannot be ruled out and, furthermore, the data presented cannot exclude the possibility that DNA adducts might arise in a small, but polymorphic-susceptible group of the population. In contrast to what their name implies, currently available anti-oestrogens exhibit either oestrogen antagonist or agonist effects depending on the tissue type, receptor sub-type and the endogenous oestrogen milieu. It is these dual and contradictory activities that may be the key to both the positive and negative long-term effects of anti-oestrogens. By competitively occupying oestrogen receptors and reducing the number of receptors available for the binding of endogenous oestradiol, anti-oestrogens inhibit oestrogen-induced stimulation of DNA synthesis and cell replication. However, in some tissues, such as the uterus, so-called anti-oestrogens can lead to stimulation of the oestrogen receptor. Depending on the anti-oestrogen and the tissue, other mechanisms may also be involved; such mechanisms include changes in oncogene expression and modulation of growth factor secretion. For patients with breast cancer, the small risk to the endometrium from tamoxifen use is vastly outweighed by the therapeutic benefit, but it is in the prophylactic trial setting that the issue is perhaps more complex. From our current findings, we speculate that a genotoxic mechanism of carcinogenicity may not be responsible for the pathology seen in the uterus of some women treated with tamoxifen; other epigenetic mechanisms of carcinogenicity now bear closer investigation.

References

P.L. Carmichael et al.


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