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Elevation of ADP-ribosylation as an Indicator of Mononuclear Leucocyte Responsiveness in Breast Cancer Patients Treated with Tamoxifen

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82 women who had had surgery for removal of breast cancer were randomised during the primary care period before initiation of any chemotherapy or radiotherapy into two groups: no drug treatment (n = 40) and 20 mg tamoxifen per day for 2 years (n = 42). Mononuclear leucocyte (MNL) fractions from blood samples were collected during the first 368 days of the study and ADP-ribosylation was quantified. Tamoxifen treatment resulted in a dose-duration increase in ADP-ribosylation. This was true even after adjustment for covariates such as age, smoking habits, oestrogen use, menstruation and tumour size. These data suggest that part of the antitumour effects of tamoxifen treatment *in vivo* relates to an enhanced immune cell responsiveness, as indicated by the increased MNL ADP-ribosylation.
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INTRODUCTION

EVIDENCE CONTINUES to be collected for an association between DNA repair capacity and cancer when using mononuclear leucocytes (MNL) as the indicator cells [1–3]. Recently our laboratory has focused on the functional importance of DNA damage accumulation via inhibition of DNA repair processes in MNL. The naturally occurring reactive oxygen species H_2O_2, which is an important promoter of the carcinogenic process [4, 5], is a potent activator of poly ADP-ribosyl transferase (ADPRT) [6], an enzyme known to be involved in DNA repair, cell proliferation and differentiation [7, 8]. MNL from individuals with cancer including breast cancer, which are exposed *in vitro* to H_2O_2, have suppressed ADPRT values [6, 9]. Moreover, suppressed ADP-ribosylation can occur at least in part from reduction/oxidation (redox) imbalance due to the fact that ADPRT is a sulphydryl-containing enzyme being up- and down-regulated by reduced and oxidised cellular glutathione, respectively [10]. Because T-lymphocyte responses to polyclonal mitogenic stimulation are likewise well-known to be modulated by redox status in a similar way [11–14], a relationship between ADP-ribosylation and immune function has been strongly supported by several studies involving direct estimation of ADPRT [15–16] and by using inhibitors of ADPRT [17–19].

Breast cancer patients have elevated serum levels of oestrogenic steroids [20]. High *in vivo* levels of oestrogenic steroids whether produced endogenously, or supplied by exogenous drug supplements, are immunosuppressive [21–22]. These effects have also been confirmed by *in vitro* studies using human lymphocytes [23], and the presence of oestrogen receptors, which would be necessary to mediate oestrogenic steroid effects on lymphocytes, have been shown to exist [24–26].

Tamoxifen has antitumour properties [27–28] which have been attributed to the potent anti-oestrogenic activity of this drug [29] although other effects have been demonstrated [30]. Hence, these data have encouraged us to consider the hypothesis that tamoxifen intervention may enhance ADP-ribosylation levels in MNL by blocking the suppressive effects of high oestrogen levels, and thereby improving lymphocyte responsiveness to immunogenic stimuli. Here we report that women receiving tamoxifen treatment following surgery for invasive breast cancer have a dose-dependent amplification of ADP-ribosylation in their MNL.

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PATIENTS AND METHODS

Patients

Consecutive female individuals (n = 82), who had had surgery for removal of invasive breast cancer (0.3–11.0 cm), were randomised during the primary care period before the initiation of any chemo- or radio therapeutic programs into two groups: one group (n = 40) received no drug treatment and the other group (n = 42) was scheduled to receive 20 mg/day tamoxifen for a 2 year period but were sampled between 7–368 days. Age ranged from 32 to 80 years with a median of 62 years. Smoking (21/82), menstruation (20/82) and oestrogen use (23/82) were other factors taken into consideration for this study.

Sampling

A 20 ml heparinised blood sample (143 USP units/10 ml) was taken by anticubital puncture and transported to the laboratory for analyses within 2 h of collection. The MNL fraction was isolated from blood by conventional procedures involving density gradient centrifugation using an Isopaque-Ficoll cushion (1.077 g/ml) as already described [31]. The biochemical assays were performed and the data presented to Dr Håken Olsson for statistical analysis without any knowledge by the biochemical laboratory of the patient material.

H_2O_2 *activated ADPRT*

The procedure was adapted from the permeabilised cell technique of Berger [32] with modifications described previously [33]. Duplicate samples of 5×10^5 MNL were cultured in 0.5 ml of physiological saline supplemented with 1% platelet-

Table 1. Descriptive statistics for the various clinical parameters analysed in relation to the ADP-ribosylation levels in the mononuclear leucocytes from 82 patients with invasive breast cancer

Parameter	n	Ln H_2O_2 activated ADPRT [mean (S.D.)]	Significance (P)
Age (years)			
< 62	43	6.89 (0.69)	
⩾ 62	39	6.74 (0.68)	< 0.31
Smoking habits			
Non-smokers	61	6.72 (0.73)	
Smokers	21	7.09 (0.47)	< 0.03
Tamoxifen treatment			
0	40	6.71 ((0.72)	
1–368 days*	42	6.92 (0.65)	< 0.17
Oestrogen user			
No	59	6.81 (0.73)	
Yes	23	6.82 (0.59)	< 0.96
Menstruation			
No	62	6.82 (0.66)	
Yes	20	6.82 (0.78)	< 0.98
Tumour size			
< 2 cm	38	6.87 (0.62)	
⩾ 2 cm	44	6.77 (0.74)	< 0.49

* 41 patients received tamoxifen for 7–55 days and 1 for 368 days.

poor autologous plasma for 1 h at 37°C in the presence of a standardised dose of 100 μmol/l H_2O_2. The cells were harvested by centrifugation at 500 *g* for 10 min and then permeabilised at 4°C by suspension for 15 min in 1.5 ml of ice-cold buffer (10 mmol/l Tris–HCl, pH 7.8; 1 mmol/l EDTA; 30 mmol/l 2-mercaptoethanol; 4 mmol/l $MgCl_2$). The cytoskeletons were recovered by centrifugation and then suspended in 50 μl permeabilisation buffer, 25 μl reaction buffer (100 mmol/l Tris–HCl, pH 7.8; 120 mmol/l $MgCl_2$), and 15 μl of [^{3}H]adenine-labelled NAD^+ (1.00 GBq/mmol, diluted to final concentration of 220 μmol/l). Incubation was continued at 30°C for 15 min. The reaction was terminated by addition of sodium dodecyl sulfate (20 μl of 12.5% solution) and 0.5 ml 3 mol/l NaCl at 60°C for 10 min. $(ADP\text{-}ribose)_n$-protein complexes were precipitated with ice-cold 7% trichloroacetic acid (TCA) and then collected onto nitrocellulose filters (Millipore HAWP 02500). This assay does not distinguish between mono- and poly-ADP-ribosylation that might be present in the cytoskeletons. Hence, we have referred to the ADP-ribosylation in cytoskeletons as ADPRT. The data were recorded as cpm TCA precipitable (ppt) [^{3}H]adenine-labelled NAD^+ per 5×10^5 cells in the presence of 100 μmol/l H_2O_2 (i.e. activated levels). The counting efficiency was 56%. The H_2O_2 activated values were converted to the Ln values for statistical analyses in order to minimise influences from non-parametric distributions.

RESULTS

The clinical parameters and descriptive statistics which were taken into consideration for analysis of the factors that influence ADP-ribosylation levels in MNL of breast cancer patients are presented in Table 1. There was essentially no evidence for any effects of age, tumour size, menstruation or oestrogen use on MNL ADP-ribosylation for the 82 women with breast cancer included in this study. However, smokers ($n = 21$) had significantly elevated ADP-ribosylation levels when compared with non-smokers ($n = 61$), and tamoxifen treatment also tended to increase ADP-ribosylation but the difference was not significant ($P < 0.17$).

Because smokers and tamoxifen treatment both increased ADPRT activity, adjustment for covariation of these factors as well as the other factors presented in Table 1 was accomplished by multiple regression analysis. Even after adjustment, oestrogen use, menstruation and tumour size did not significantly influence the ADPRT activity and so they were not included any further in the analysis. However, both smoking habits and tamoxifen treatment became significant influencing factors on ADPRT activity after adjustment for covariates and the inclusion of a smoking/tamoxifen interaction term into the statistical model (Table 2). In an effort to pursue the understanding of the influence of smoking on the evaluation of the effects tamoxifen treatment has on ADPRT activity, the sample was split into smokers and non-smokers and again analysed by multiple regression analysis. Now, non-smokers receiving tamoxifen had significantly elevated ADPRT levels when compared with the no tamoxifen treated group, whereas no significant effect on ADPRT activity could be shown among smokers (Table 2). However, there was a significant negative influence of age on ADPRT activity among smokers after adjustment for tamoxifen treatment. These data support the interpretation that smokers, who already have elevated ADPRT activity ($\beta = 0.66$, $P < 0.007$, Table 2), are not sensitive to any further elevation of ADPRT activity by tamoxifen treatment, and this lack of effect may be age influenced.

The number of days that the breast cancer patients received tamoxifen was also recorded which has permitted the evaluation of this parameter as a continuous variable. The analysis of MNL ADPRT activity with regard to tamoxifen treatment, when it was considered as a time variable adjusted for age and smoking habits for the total sample and for non-smokers, still remained statistically significant (Table 3). Likewise, this was true whether the no tamoxifen treatment group was included ($n = 61$, $\beta = 0.005$, $P < 0.01$) or not ($n = 34$, $\beta = 0.004$, $P < 0.03$) in the regression analysis.

The strength of the association between ADPRT activity in MNL of non-smoking breast cancer patients and the duration of their treatment with tamoxifen is presented in Fig. 1. A

Table 2. Multiple regression analysis of the clinical parameters influencing the ADP-ribosylation levels in mononuclear leucocytes from breast cancer patients

Parameter	β-Coefficient	Significance (P)
Total sample ($n = 82$)		
Age (32–80 years)	−0.004	< 0.56
Smoking habits	0.66	< 0.007
With or without tamoxifen treatment	0.39	< 0.02
Interaction (tamoxifen/smoking)	−0.53	< 0.12
Non-smokers ($n = 61$)		
Age (32–80 years)	0.003	< 0.72
With or without tamoxifen treatment	0.44	< 0.02
Smokers ($n = 21$)		
Age (32–80 years)	−0.02	< 0.05
With or without tamoxifen treatment	−0.25	< 0.31

Table 3. The effect of duration of tamoxifen treatment in days on the ADP-ribosylation levels in the mononuclear leucocytes from breast cancer patients

Parameter	β-Coefficient	Significance (*P*)
Total sample (n = 82)		
Age (32–80 years)	−0.003	< 0.67
Smoking	0.57	< 0.01
Time on tamoxifen (0–368 days)	0.005	< 0.009
Interaction (tamoxifen/smoking)	−0.35	< 0.27
Non-smokers (n = 61)		
Age (32–80 years)	0.004	< 0.69
Time on tamoxifen (0–368 days)	0.005	< 0.01
Non-smokers (n = 34)		
Age (32–80 years)	−0.017	< 0.16
Time on tamoxifen (1–368 days)	0.004	< 0.03

significant linear correlation (r = 0.42, $P < 0.01$) was established which has emphasised a dose-duration effect of tamoxifen within the first 55 days of treatment.

DISCUSSION

This study has demonstrated a time-dependent increase in MNL ADPRT activities from breast cancer patients receiving adjuvant tamoxifen therapy (Fig. 1). The key issue for discussion is whether there are any possible health consequences relating to the elevated levels of this enzymatic activity in MNL. DNA strand breaks are required for activation of ADPRT [7] and for lymphocyte proliferation responses [17]. Cancer patients [6] including women with breast cancer [9] have suppressed MNL ADPRT activity which in turn has been shown to signal a reduced lymphocyte proliferative capacity of importance to immune function [15]. Immunosuppression among women with breast cancer has been reported, and it was attributed to a defective production of interleukin-2 receptors in peripheral blood lymphocytes [34]. Although studies with tamoxifen have shown no significant change in activation of the lymphocyte proliferative response to phytohaemagglutinin [35, 36], natural killer cell activity has been shown to be enhanced *in vitro* [37]. Hence, the evidence so far supports the interpretation that tamoxifen treatment may improve immune cell function and this effect is detected by elevated ADPRT activity in MNL. Regardless, because suppressed MNL ADPRT activity has been consistently associated with the presence of cancer [6, 9], and even with conditions that predispose to cancer (e.g. ulcerative colitis and adenomatous polyps as risk factors for colon cancer) [38, 39], the raising of ADP-ribosylation by tamoxifen intervention has modulated this biologic indicator toward lower cancer risk values.

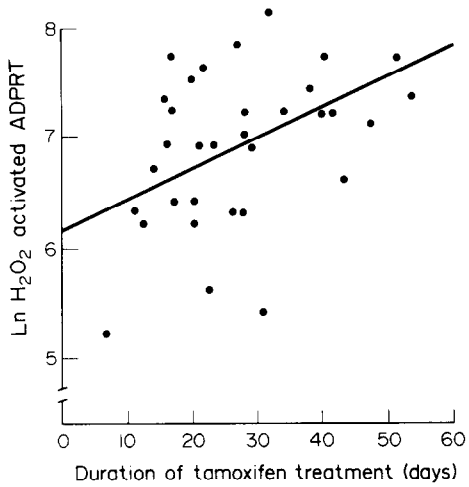

Fig. 1. A linear correlation between duration of tamoxifen treatment (20 mg/day) in days and the ADP-ribosylation levels in mononuclear leucocytes of non-smoking women with breast cancer. The data shown are unadjusted values and the case having 368 days on tamoxifen was excluded giving $Y = 0.025x + 6.19$, $r = 0.42$, $P < 0.01$. The significance of the correlation remained even if the 368-days case or the zero-tamoxifen treatment group were included in the analysis ($Y = 0.017x + 6.44$, $r = 0.40$, $P < 0.03$).

Another general conclusion found in the scientific literature relating to ADP-ribosylation levels has been that, as cells proceed down the pathway toward differentiation and lose their ability to respond to growth stimuli, their ADPRT activities progressively decrease [40, 41]. These data provide yet another strong parallel to this study where the raising of ADP-ribosylation by tamoxifen intervention was found (Table 3, Fig. 1). For example, MNL having high ADPRT activity would be expected to have a high degree of proliferative response to growth stimuli, which for the lymphocytes present in the MNL, would in turn be linked to an important immune cell function (i.e. proliferation [15–19]).

To our knowledge, any previous evidence that tamoxifen has effects *in vivo* on either MNL or immune cell function has not been demonstrated. The most commonly referred to antitumour mechanism for tamoxifen relates to its anti-oestrogenic properties and the subsequent steroidal effects on target tissue proliferation [27–29]. If tamoxifen acted as an anti-oestrogen, this would require the presence of oestrogen receptors, but some oestrogen receptor-negative breast cancers respond to tamoxifen [42]. Based on the data reported in this study, an attractive alternative mechanism, which could explain this discrepancy, is that tamoxifen possesses immunostimulatory properties via its effects on MNL ADP-ribosylation and cell proliferation. This hypothesis is fortified by the fact that lymphocytes have oestrogen receptors [24–26] which could be potentially blocked by tamoxifen [29]. Elevated levels of oestrogens are immunosuppressive [21–23] and breast cancer patients have high serum levels of oestrogens [20]. These conditions are necessary prerequisites for an anti-oestrogenic explanation of immune cell stimulation to be possible. An alternative explanation that is equally consistent with tamoxifen being an immunostimulatory agent comes from a recent observation where granulocyte production of pro-oxidants such as H_2O_2 is inhibited by physiologically relevant doses of tamoxifen [43]. Hydrogen peroxide in the presence of granulocytes or myeloperoxidase and chloride ions produce chloramines which can irreversibly inhibit MNL ADP-ribosylation and suppress immune cell function (Pero *et al.*, unpublished data).

It is generally known that cigarette smoke contains hundreds of agents that produce DNA damage *in vivo* in peripheral lymphocytes and cause immunotoxicity [44]. Our laboratory has observed previously [6] as well as in this study (Table 2) that smokers have increased MNL ADPRT activity. There was also a strong interaction between tamoxifen treatment and smoking which interfered with the analysis of ADPRT in this study (Tables 2 and 3). These data can be explained if one considers

that the DNA damaging effects coming from inhaled smoke indiscriminately activate ADPRT and produce immunosuppression by tamoxifen-insensitive mechanisms, thereby masking or blocking any effects coming from tamoxifen treatment. This interpretation would predict that smokers receiving tamoxifen treatment might have a different prognosis than non-smokers. This point is currently under investigation by our group.

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