Pergamon

European Journal of Cancer Vol. 32A, No. 3, pp. 413–416, 1996

Printed in Great Britain
0959-8049/96 $15.00+0.00

0959-8049(95)00517-X

Original Paper

Short-term Effects of Pure Anti-oestrogen ICI 182780 Treatment on Oestrogen Receptor, Epidermal Growth Factor Receptor and Transforming Growth Factor-alpha Protein Expression in Human Breast Cancer

R.A. McClelland,[1] J.M.W. Gee,[1] A.B. Francis,[1] J.F.R. Robertson,[2] R.W. Blamey,[2] A.E. Wakeling[3] and R.I. Nicholson[1]

[1]Tenovus Cancer Research Centre, Cardiff; [2]City Hospital, Nottingham; and [3]Zeneca Pharmaceuticals, Macclesfield, UK.

Expression of oestrogen receptor (ER), epidermal growth factor receptor (EGFR) and transforming growth factor-α (TGFα) proteins was assessed by immunocytochemistry on primary breast cancer specimens obtained before and following short-term (7-day) presurgical exposure to pure anti-oestrogen (7α- [9- (4,4,5,5,5-pentafluoropentylsulphinyl) nonyl] estra-1,3,5, (10)-triene-3,17 β-diol, ICI 182780) treatment and compared with no-treatment controls. Paired needle-core and mastectomy samples were obtained from 21 patients. Effects of ICI 182780 (10^{-7}M) on MCF7 breast cancer cell ER, EGFR and TGFα expression were also examined over 14 days. ER protein was significantly suppressed by ICI 182780 *in vivo* ($P = 0.009$) and comparative analysis of short term ICI 182780 effects *in vitro*, using ER-positive MCF7 cells, gave largely equivalent results. EGFR and TGFα protein levels were unaltered by treatment. ICI 182780 suppresses ER without a concomitant rise in either EGFR or TGFα.

Key words: human breast cancer, pure anti-oestrogens, oestrogen receptor, epidermal growth factor receptor, transforming growth factor-alpha
Eur J Cancer, Vol. 32A, No. 3, pp. 413–416, 1996

INTRODUCTION

THE INVOLVEMENT of oestrogen in the growth and development of breast cancer has led to the formulation of a number of anti-endocrine treatments. Of these, the non-steroidal anti-oestrogen tamoxifen has probably been most widely used in the treatment of postmenopausal breast cancer [1]. Unfortunately, most patients become resistant to tamoxifen following prolonged exposure and experience disease progression [2, 3]. The current clinical evaluation of the recently developed 'pure' anti-oestrogens [4] is therefore being viewed with interest. These compounds, typified by ICI 182780 {7α- [9-(4,4,5,5,5-pentafluoropentylsulphinyl) nonyl) estra-1,3,5, (10)-triene-3,17 β-diol}, have no agonistic activity *in vitro* and are more potent inhibitors of tumour cell growth than existing treatments [5, 6]. They are also reported to inhibit the growth stimulatory effects of insulin-like growth factor (IGFI) and transforming growth factor-α (TGFα) [7], and reduce interactions between oestrogen receptors (ER) and EGF/TGFα-EGFR signalling *in vitro* [7]. Furthermore, these compounds are associated with a rapid loss of ER, and of oestrogen-regulated gene expression *in vivo* [8], an important consideration with respect to the inverse relationship between the expression of EGFR (epidermal growth factor receptor) and ER proteins in clinical specimens [9]. It is also significant that tumours enriched for the presence of EGFR and/or TGFα are recognised to be frequently insensitive to endocrine therapy [9, 10], and that *in vitro* studies have shown a number of compounds (e.g. TPA [11], sodium butyrate [12]) capable of suppressing ER but resulting in a concomitant rise in EGFR. These findings have led to some concern regarding the possibility that ICI 182780 might therefore directly or indirectly, promote EGFR and/or TGFα expression and hence favour the development of endocrine insensitivity. We address this issue through the comparative use of immunocytochemistry on sections from pre- and posttreatment clinical breast cancers, and on cultured MCF7 breast cancer cells.

Correspondence to R.I. Nicholson.
Revised 11 Aug. 1995; accepted 15 Sep. 1995.

EJC 32:3-C

PATIENTS AND METHODS

Patients and clinical data

Tumour samples were obtained from 21 breast cancer patients entered into a phase 1 clinical trial of the pure anti-oestrogen ICI 182780 given for a short period (7 days) prior to the surgical removal of the cancer [8, 13]. Samples included in the current report were all from postmenopausal patients presenting to the breast clinic of the City Hospital, Nottingham, with histologically verified primary breast cancer and no evidence of metastasis. Patients giving their informed consent were randomised to a no pre-operative treatment group ($n = 7$), or treatment groups of (a) 6 mg (low dose) ($n = 8$), or (b) 18 mg (high dose) ($n = 6$) daily intramuscular (i.m.) injections of ICI 182780 for 7 days prior to surgery.

Pre-anti-oestrogen treatment tumour samples were obtained by needle core biopsy. Post-treatment tumour samples were obtained at the time of primary breast surgery.

Upon excision, representative samples of tumour specimens were snap frozen on dry ice and stored at −70°C. Portions of these frozen tissues were mounted for cryostat sectioning in a suitable embedding medium and sections prepared and fixed for immunocytochemistry.

Tissue culture

ER-positive MCF7 breast cancer cells were subcultured on to sterile cover slips in phenol red-free RPMI medium containing 5% dextran-coated charcoal-stripped fetal calf serum (day 0). Cells were then either maintained in this steroid-depleted environment (−E2) for up to a further 14 days or with the addition of 10^{-7} M ICI 182780 (+182) after 24 h (day 1), replenished every 2 days. Representative coverslips were removed, initially at day 2 and thereafter at the time points illustrated in Figure 2, and fixed for immunocytochemistry.

Immunocytochemistry methodologies

The methodologies for ER immunocytochemical assay (ICA) (monoclonal ER-ICA kit, Abbott Diagnostics Inc., Chicago, Illinois, U.S.A.) for EGFR-ICA (monoclonal EGFR1 antiserum, Amersham plc, Amersham, U.K.) and TGFα-ICA (monoclonal TGFα1, Oncogene Science Inc., Uniondale, New York, U.S.A.) were performed, for both sections and coverslips, as previously described [14, 9, 10, respectively]. Previous publications have also established the validity of the manual slide assessment method used, and of the Hscore calculation as a means of summarising both percentage of tumour cells staining and intensity of stain data [10, 15]. The calculation is extended here to include assessment of EGFR-ICA results in an identical manner. An assay cutoff for positivity of ≥ 0.02 Hscore units is assumed for all assays and represents a minimum of 2% of tumour cells demonstrating weak, but specific immunostaining [10, 15].

RESULTS

Twenty one paired pre- and post-ICI 182780 treatment specimens were included within this study. ER-ICAs were assessible on 19, EGFR-ICAs on 19 and TGFα-ICAs on 15.

Twelve of 19 (63%) pretreatment samples were ER-ICA positive (Hscores ≥ 0.02). The effects of short-term treatment on the expression of this protein are presented in Figure 1(a). No significant differences were observed between pre- and post-treatment ER-ICA results for the no-treatment (control) group (Mann–Whitney $P = 1.00$ n.s). Similarly, no significant

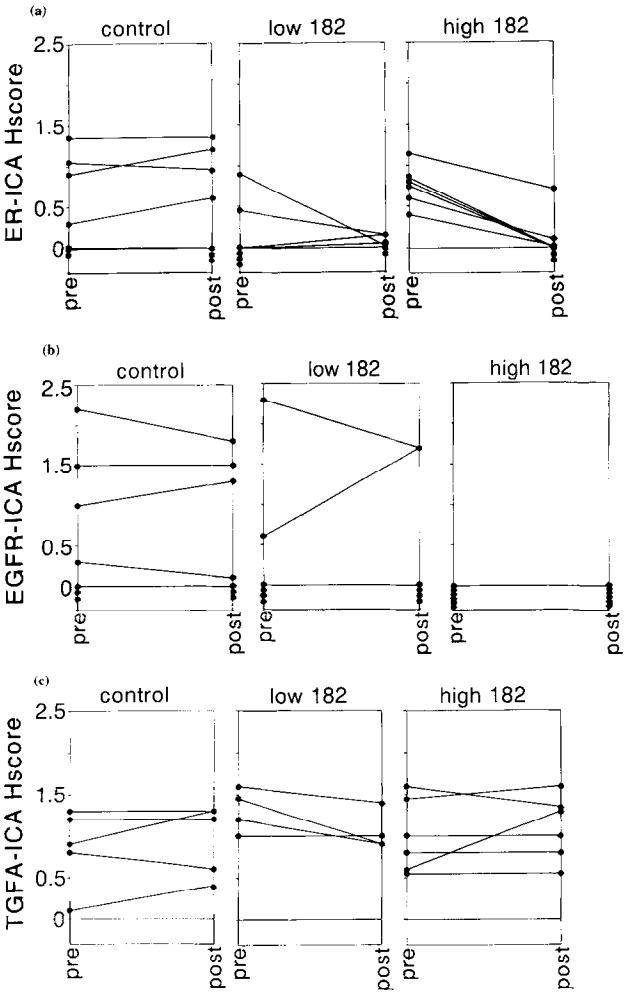

Figure 1. The changes in immunocytochemical staining Hscore values are illustrated for samples from individual patients which were obtained 7 days pre (pre) and at primary breast cancer surgery (post). During this period patients either received no treatment (control), or daily i.m. 6 mg dose (low 182) or 18 mg dose (high 182) ICI 182780 treatment. Figures showing changes in (a) ER-ICA, (b) EGFR-ICA and (c) TGFα-ICA are presented.

change in ER was observed in those tumours treated with 6 mg/day ICI 182780 (low 182) (Mann–Whitney $P = 0.818$ n.s). Since four of six of these pretreatment samples were ER-negative, no relevant conclusions can be drawn from this group. It is, however, noteworthy that ER levels fell sharply following low dose ICI 182870 treatment of the two ER-positive pretreatment samples in this group. A significant effect on ER protein was observed following 18 mg/day (high 182) ICI 182780 where levels fell in 6/6 post-treatment samples (Mann–Whitney $P = 0.009$). Post-treatment ER expression in samples which were pretreatment ER-negative was not significantly affected by ICI 182780. Positive pre- and post-treatment EGFR-ICAs (Hscore ≥ 0.02) were recorded in 6/19 (32%) samples studied and a significant inverse correlation between pretreatment ER and EGFR-ICA results was observed within this data, with 10/16 samples ER+/EGFR- and 5/16 ER−/EGFR+ ($P = 0.005$). Figure 1(b) illustrates the effects of treatment on EGFR protein expression. EGFR staining did not significantly differ between pre- and post-treatment results in either the untreated control group or in

ICI 182780 groups at either dose (Mann–Whitney $P = 1.00$ in all cases), irrespective of pretreatment ER status. Although only 2 patients treated with ICI 182780 had initially EGFR positive tumours, in both cases levels were similar post-treatment. Importantly, all (6/6) high dose samples which were EGFR- pre ICI 182780 treatment (and were ER+) remained so following treatment. Since no high dose ICI 182780 pre-treatment samples were EGFR positive, it is not possible to comment on the effects of this dose on elevating existing levels of EGFR.

Immunodetectable levels of TGFα protein were observed in all pre- and post-treatment samples studied (IHscore ≥ 0.02) (Figure 1c). Ranges and median values for all groups were similar and no significant trend between differences were found in any group (Mann–Whitney P values: 0.691 control, 0.114 low 182 and 0.818 high 182).

Figure 2(a) compares the effects of the addition of 10^{-7} M ICI 182780 to oestrogen-withdrawn ER+ MCF7 breast cancer cells with cells maintained in unsupplemented −E2 conditions. A marked time-dependent loss of immunostainable MCF7-ER followed the addition of ICI 182780 with levels virtually undetectable after day 4. In contrast, a steady increase in ER levels was observed in the −E2 control group throughout the study period.

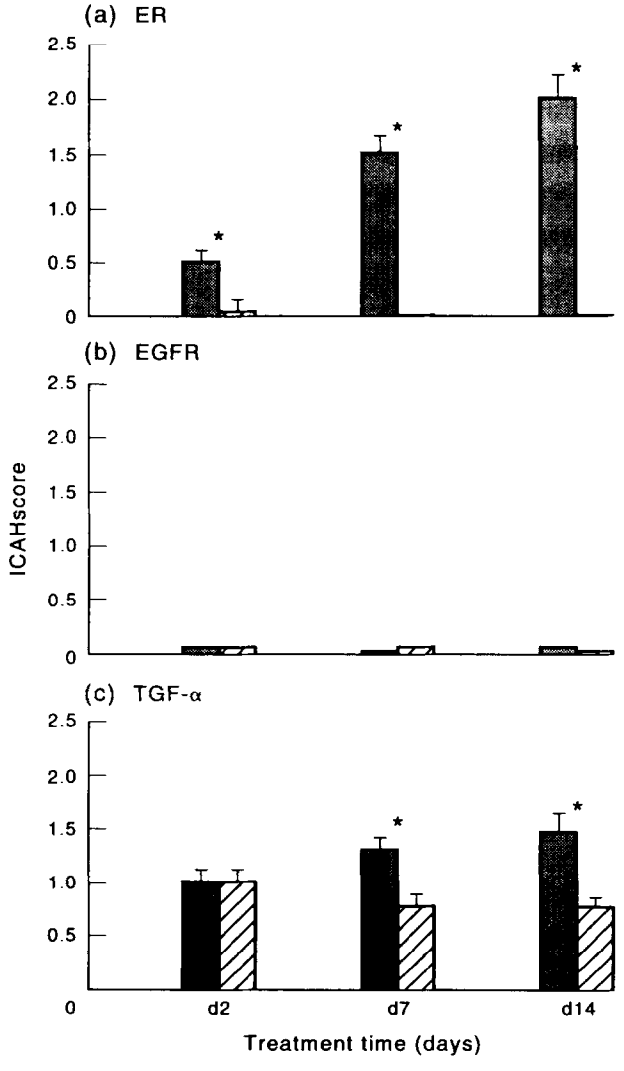

Figure 2. The effects of oestrogen-depleted (−E2) (▒) versus ICI 182780-supplemented (▨) medium on the growth of MCF7 breast cancer cells. Vertical bars represent +/− 1 S.D. *Significant difference between −E2 and +182 groups at this time point (Mann–Whitney $P < 0.005$).

Very low levels of immunostainable EGFR were expressed by MCF7 cells and these levels were maintained throughout in oestrogen-withdrawn conditions (Figure 2). The addition of ICI 182780 caused no change in these low levels. The effects of −E2 withdrawal and ICI 182780 on TGFα expression in MCF7 cells are portrayed in Figure 2(c). A sustained rise in TGFα levels of −E2 withdrawn cells was observed throughout the 14 day period, although at a lower rate of increase than ER. Conversely, ICI 182780 appeared to suppress TGFα, but again less effectively than for ER, with readily detectable levels of TGFα still being present despite 13 days of exposure to ICI 182870.

DISCUSSION

This study represents the first immunocytochemical analysis of the effects of short-term pure anti-oestrogen treatment on the expression of EGFR and its ligand TGFα *in vivo*. Levels of these proteins and of ER in untreated presurgical needle-core biopsies were compared with those in the parent tumour removed at mastectomy following 7 days exposure to the drug at either of two doses.

Results show that, as previously reported in a larger series, from which the current study samples were derived, ER protein levels were significantly suppressed by 18 mg/day ICI 182780 treatment [8, 13]. In contrast, no apparent alteration in EGFR protein expression was observed *in vivo*, with pre-treatment EGFR−ve tumour samples remaining so and both EGFR+ves unchanged. Thus, neither a reduction in level or a total loss of tumour ER following ICI 182780 treatment was accompained by an associated increase in EGFR.

These observations are important in view of the established inverse correlation between ER and EGFR protein expression in human breast cancer, a feature also observed in this study, and the loss of endocrine sensitivity which accompanies an elevation in EGFR and TGFα levels. [9].

Importantly, we [16] and others [17] have previously found ER mRNA levels to be unaltered by short term pure anti-oestrogen treatment *in vivo*, despite a fall in ER protein with ICI 182780, suggestive of the drug's effects being elicited by the formation of a drug–ER-complex which is rapidly denatured [18]. These observations and the discovery that a number of chemicals can simultaneously cause the rapid suppression of ER mRNA levels and increase in EGFR mRNA [11, 12] suggest that changes in ER protein in the absence of effects on ER mRNA are insufficient to promote alterations in EGFR gene expression.

Although ICI 182780 effects on ER and EGFR expression *in vitro* essentially parallel those observed *in vivo*, levels of TGFα protein by contrast, were not influenced *in vivo* but significantly suppressed in tissue culture experiments by ICI 182780. Whilst data regarding the role of oestrogens in the regulation of TGFα *in vivo* are inconclusive, TGFα is recognised as being oestrogen inducible *in vitro* and acts as a mitogen for breast cancer cells [19]. It has also been shown to partially override the inhibitory activity of tamoxifen [20]. Consequently, the suppression of TGFα by ICI 182780 *in vivo* would represent a favourable characteristic were it to be reflected in clinical breast cancer specimens. It seems possible that differences between clinical and *in vitro* data may reflect an increased sensitivity of MCF7 cells to the drug following a

brief period of oestrogen withdrawal, and that the *in vitro*-observed effects might therefore be achieved clinically following longer-term exposure to the drug.

The TGFα/EGFR growth factor signalling pathway has been implicated in the development of endocrine resistance, probably through a loss of the normal signal transduction mechanisms which control the interactivity between oestrogen and growth factors. It is therefore important that this study presents no evidence that treatment with pure anti-oestrogens *in vivo* or *in vitro* results in increased EGFR or TGFα expression which might promote an immediate change in the endocrine sensitivity of breast tumours.

1. Jordan VC. A current view of tamoxifen for the treatment and prevention of breast cancer. *Br J Pharmacol* 1993, **110**, 507–517.
2. Wolf DM, Jordan VC. Drug resistance to tamoxifen during breast cancer therapy. *Br Cancer Res Treat* 1993, **27**, 27–40.
3. Osborne CK, Coronado E, Allred DC, Wiebe V, DeGregorio M. Acquired tamoxifen resistance: correlation with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxytamoxifen. *J Natl Cancer Inst* 1991, **83**, 1477–1482.
4. Wakeling AE. The future of pure antiestrogens in clinical breast cancer. *Br Cancer Res Treat* 1993, **25**, 1–9.
5. Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. *Cancer Res* 1991, **51**, 3867–3873.
6. Wakeling AE, Bowler J. Novel antiestrogens without partial agonist activity. *J Steroid Biochem* 1988, **31**, 645–653.
7. Nicholson RI, Francis AB, McClelland RA, Manning DL, Gee JMW. Pure anti-oestrogens (ICI 164384 and ICI 182780) and breast cancer: is the attainment of complete oestrogen withdrawal worthwhile? *Endocrine-related Cancer* 1994, **3**, 1–13.
8. Nicholson RI, Gee JMW, Eaton CL, *et al.* Pure anti-estrogens in breast cancer: experimental and clinical observations. In Motta M, Serio M, eds. *Sex Hormones and Anti-hormones in Endocrine Dependent Pathology: Basic and Clinical Aspects*. Amsterdam, Elsevier Science, 1994, 347–360.
9. Nicholson RI, McClelland RA, Gee JMW, *et al.* Epidermal growth factor expression in breast cancer: association with response to endocrine therapy. *Br Cancer Res Treat* 1994, **29**, 117–125.
10. Nicholson RI, McClelland RA, Gee JMW, *et al.* Transforming growth factor-α and endocrine sensitivity in breast cancer. *Cancer Res* 1994, **54**, 1684–1689.
11. Lee CSL, Koga M, Sutherland RL. Modulation of estrogen receptor and epidermal growth factor receptor mRNAs by phorbol ester in MCF7 breast cancer cells. *Biochem Biophys Res Commun* 1989, **162**, 415–421.
12. deFazio A, Chiew Y-E, Donoghue C, Lee CL, Sutherland RL. Effect of sodium butyrate on estrogen receptor and epidermal growth factor receptor gene expression in human breast cancer cell lines. *J Biol Chem* 1992, **25**, 18008–18012.
13. Defriend DJ, Howell A, Nicholson RI, *et al.* Investigation of a new pure antiestrogen (ICI 182780) in women with primary breast cancer. *Cancer Res* 1994, **54**, 408–414.
14. Walker KJ, Bouzubar N, Robertson J, *et al.* Immunocytochemical localization of estrogen receptor in human breast tissue. *Cancer Res* 1988, **48**, 6517–6522.
15. McClelland RA, Finlay P, Walker KJ, *et al.* Automated quantitation of immunocytochemically localized estrogen receptors in human breast cancer. *Cancer Res* 1990, **50**, 3455–3550.
16. McClelland RA, Manning DL, Gee JMW, *et al.* Effects of short-term antiestrogen treatment of primary breast cancer on estrogen receptor mRNA and protein expression and on estrogen-regulated genes. *Br J Cancer Treat*, in press.
17. Gibson MK, Nemmers LA, Beckman LA, Davis VL, Curtis SW, Korach KS. The mechanism of ICI 164384 antioestrogenicity involves rapid loss of estrogen receptor in uterine tissue. *Endocrinology* 1991, **129**, 2000–2010.
18. Fawell SE, White R, Hoare S, Sydenham M, Page M, Parker MG. Inhibition of estrogen receptor DNA binding by the pure antiestrogen ICI 164384 appears to be mediated by receptor dimerization. *Proc Natl Acad Sci USA* 1990, **87**, 6883–6887.
19. Lippmann ME, Dickson RB, Gelmann EP, *et al.* Growth regulatory peptide production by human breast cancer cells. *J Steroid Biochem* 1988, **30**, 53–61.
20. Koga M, Sutherland RL. Egf can partially reverse the effects of antiestrogens. *Biochem Biophys Res Commun* 1987, **146**, 738–745.

Acknowledgement—We gratefully acknowledge the support given for this work by the Tenovus Organisation.