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Alterations in the insulin-like growth factor system during treatment with diethylstilboestrol in patients with metastatic breast cancer

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Summary Alterations in the insulin-like growth factor (IGF)-system were evaluated in 16 patients treated with diethylstilboestrol 5 mg 3 times daily. Fasting blood samples were obtained before treatment and after 2 weeks, 1 month and/or 2–3 months on therapy. Insulin-like growth factor (IGF)-I, IGF-II, free IGF-I, IGF-binding protein (IGFBP)-1, IGFBP-2 and IGFBP-3 were measured by radioimmuno-/immunoradiometric-assays. All samples were subjected to Western ligand blotting as well as immunoblotting for IGFBP-3. We observed a significant decrease (percentage of pretreatment levels with 95 confidence intervals of the mean) in IGF-I [2 weeks 63% (49–79); 1 month 56% (44–73); 2–3 months 66% (53–82)], IGF-II [2 weeks 67% (56–80); 1 month 60% (52–68); 2–3 months 64% (55–75)], free IGF-I [2 weeks 29% (19–42); 1 month 25% (18–36); 2–3 months 31% (21–46)], IGFBP-2 [2 weeks 53% (18–156); 1 month 69% (61–78); 2–3 months 66% (57–78)], IGFBP-3 [2 weeks 74% (63–85); 1 month 69% (62–76); 2–3 months 71% (63–80)], as well as IGFBP-3 protease activity [2 weeks 71% (54–95); 1 month 78% (64–94); 2–3 months 71% (54–93)]. Contrary, the plasma levels (percentage of pretreatment levels with 95 confidence intervals of the mean) of IGFBP-1 [2 weeks 250% (127–495); 1 month 173% (138–542); 2–3 months 273% (146–510)] and IGFBP-4 [2 weeks 146% (112–192); 1 month 140% (116–169); 2–3 months 150% (114–198)] increased significantly. While this study confirms previous observations during treatment with oral oestrogens in substitution doses, the reduction in plasma IGF-II, free IGF-I, IGFBP-2 and -3 are all novel findings. A profound decrease in free IGF-I suggests a reduced bioavailability of IGFs from plasma to the tissues. These observations may be of significance to understand the mechanisms of the antitumour effect of diethylstilboestrol in pharmacological doses. © 2001 Cancer Research Campaign http://www.bjcancer.com

Keywords: high dose oestrogens; breast cancer therapy; IGF-system

Endocrine therapy has a key role in the treatment of metastatic breast cancer. While oestrogens given in pharmacological doses have been known for decades to cause antitumour effects (Haddow et al, 1944; Carter et al, 1977), they were replaced by tamoxifen in the late 1970s. The reason to this was the lower toxicity of the antioestrogen, while the 2 regimens were considered to be equally effective with respect to tumour response (Ingle et al, 1981). Long-term follow-up results have suggested diethylstilboestrol to be more effective than tamoxifen (Peethambaram et al, 1999).

The mechanisms by which oestrogens in high doses cause anti-tumour effects in breast cancer are not known. However, in vitro studies have focused on the importance of cross-talk between steroids and growth factors, in particular the insulin like growth factors (IGFs), to breast cancer growth (Westley and May, 1994; Lee et al, 1997).

IGF-I is a potent mitogen to breast cancer cell lines in vitro (Karey and Sirbasku, 1988) and high plasma levels of IGF-I has been reported to be a risk factor for breast cancer development in premenopausal women (Hankinson et al, 1998). Inhibition of IGF-I receptor activation by antibodies or antisense strategies has been shown to cause antitumour effects in animal models (Arteaga et al, 1989; Dunn et al, 1998). IGF-I and -II are expressed by many different tissues and may thus act by both paracrine and autocrine mechanisms in addition to the large circulating plasma pool of peptides (Daughaday and Rotwein, 1989). In addition, the bioavailability of IGF-I as well as IGF-II to the tissues may be influenced by plasma levels of the 6 different IGF-binding proteins (IGFBPs), IGFBP related proteins, phosphorylation status of the IGFBPs as well as IGFBP-protease activity (Jones and Clemmons, 1995; Hwa et al, 1998). About 99% of IGF-I and IGF-II are found in a ternary complex consisting of IGF-I or -II, IGFBP-3 (the major IGFBP in plasma) and an acid labile subunit (ALS). Thus, only a minor fraction of circulating IGFs is free (or easily dissociable) and therefore available to the tissues. Release of IGF-I from the circulating pool may be facilitated through increased activity of IGFBP-3 proteases, reducing the binding affinity of IGF-I to the ternary complex (Lassarre and Binoux, 1994). Increased IGFBP-3 protease activity is observed in several clinical conditions including advanced breast cancer (Giudice et al, 1990; Müller et al, 1993; Bereket et al, 1995; Cotterill et al, 1996; Frost et al, 1996).

Previous studies have revealed oral administration of oestrogens for hormone substitution (Weissberger et al, 1991; Helle et al, 1996c) as well as the SERMS tamoxifen and droloxifene (Colletti et al, 1989; Lønning et al, 1992; Helle et al, 1996a) to cause a moderate reduction in total IGF-I but elevated IGFBP-I. However, the effect of oestrogens in pharmacological doses on the IGF-system has not been reported previously.

To evaluate potential alterations in the IGF-system in breast cancer patients receiving oestrogens in pharmacological doses, we

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measured IGF-I and IGF-II together with the IGF-binding proteins and IGFBP-3 protease activity in 16 patients suffering from metastatic breast cancer before and during treatment with diethylstilboestrol 5 mg 3 times daily.

SUBJECTS AND METHODS

Patients

16 postmenopausal women with progressive metastatic breast cancer failing multiple endocrine regimens were enrolled in a phase II study evaluating treatment with diethylstilboestrol 5 mg 3 times daily. The median age was 72 years (range 52–87), and the number of previous endocrine regimens ranged from 3 to 10 (median 4). Last treatment before diethylstilboestrol therapy and the length of the washout period (time between last dose of their previous therapy and pre-treatment blood sampling) is given in Table 1. The clinical results are to be reported elsewhere as a 2-centre study (Lønning et al, 2001). The study was approved by the regional ethical committee.

Blood sampling

Fasting blood samples (heparinized vials) were obtained before treatment and after 2 weeks, 1 month and/or 2–3 months during treatment with diethylstilboestrol. Samples were centrifugated within 20 minutes and plasma aliquots stored at –20 C until analysis.

Materials

Human recombinant IGF-I and IGF-II were purchased from GroPep (Adelaide, Australia). IGF-I and IGF-II were iodinated using the chloramine-T method. Labelled peptide was separated from non-incorporated ^{125}I by AcA 202 columns (BioSepra, Villeneuve, France) using 1 × 40 cm columns.

Assays

Plasma levels of IGF-I and IGF-II (Frost et al, 1996) were measured by RIA following acid-acetone extraction (Bowsher et al, 1991). Intra-and inter-assay coefficients of variations were 3.5% and 6.2% for IGF-I and 5.5% and 12.9% for IGF-II, respectively. Commercial kits (IRMA/RIA) for free IGF-I, IGFBP-I, IGFBP-2, and IGFBP-3 were purchased from Diagnostic System Laboratories (Webster, TX), and the measurements were made according to the manufacturer's instructions.

The IGFBP profile in the plasma was analysed by Western ligand blotting (WLB) using a modified version (Coulson et al, 1991) of the technique originally developed by Hossenlopp (Hossenlopp et al, 1986). ^{125}I labelled IGF-I and IGF-II was used as tracer binding IGF-binding proteins on the membrane. Radiolabelled IGFBPs were visualized by autoradiography and quantified using a densitometric scanner (Pharmacia LKB, Uppsala, Sweden). The IGFBP pattern was compared with the profile of a normal plasma pool (NP), and samples from each patient were analysed in the same run for comparison. In order to analyse for IGFBP-3 proteolysis, the membranes were immunoblotted (after WLB) using a polyclonal antiserum against IGFBP-3 purchased from Diagnostic Systems Laboratories (Webster, TX) at a final dilution of 1:10 000. The membranes were then developed (showing intact IGFBP-3 as well as fragments) using enhanced chemiluminescent reagents supplied by Amersham (Aylesbury, UK) according to the manufacturer's instruction, and the films subjected to densitometric scanning. IGFBP-3 protease activity was measured indirectly as IGFBP-3 fragmentation, defined as the ratio of the major IGFBP-3 fragment (30kDa) to total IGFBP-3 evaluated by densitometric scanning of immunoblots.

Statistics

Testing for distribution of the different IGF parameters with Q–Q plots in our normal populations of pre- and postmenopausal women revealed all parameters to be best fitted to a log normal distribution with the exception of IGFBP-3 protease activity. This parameter was best described by a normal distribution. Thus,

Table 1 Demographic and clinical data of patients included in the study

Patient no.	Age (years)	No. of previous endocrine regimens	Last treatment before DES	Response to DES	Washout period (days)
1	86	3	Formestane	CR	30
2	53	6	Formestane	PD	8
3	65	3	Tamoxifen	PR	47
4	73	4	Exemestane	PD	14
5	74	5	Anastrozole	PR	14
6	76	5	Tamoxifen	SD	27
7	71	4	Anastrozole	PD	30
8	70	10	Anastrozole	SD	21
9	62	3	MA	PR	21
10	70	5	Formestane	PD	24
11	79	3	MA + Formestane	CR	14
12	87	5	Tamoxifen	PR	42
13	80	5	Tamoxifen	CR	791
14	72	5	Tamoxifen	SD	19
15	72	3	MA	CR	30
16	68	3	Aminoglutethimide	PR	36

Response was evaluated according to the UICC criteria; CR = complete response, PR = partial response, SD = stable disease, > 3 months, PD = progressive disease. MA = megestrol acetate. Washout period = time period from stopping last endocrine regimen to commencement of diethylstilbestrol.

Table 2 Values of IGF-I, F-IGF-I, IGF-II, IGFBP-1, IGFBP-2 and IGFBP-3 measured by RIA/IRMA, and IGFBP-2, -3 and -4 by Western ligand blot before treatment, and percentage of pretreatment levels/percentage change at various intervals during treatment with diethylstilboestrol. IGFBP-3 protease activity is measured indirectly as the ratio of fragmented to total IGFBP-3 on immunoblots. Values are given as geometrical mean values (with 95% confidence intervals) with the exception of IGFBP-3 protease where arithmetic values are given. During treatment alterations in IGFBP-3 protease activity are given as percentage change, while the other parameters are given as percentage of pretreatment values

	Measured levels (nmol l^{-1}) percentage change/percentage of pretreatment levels			
	Before treatment (*n* = 16)	**2 weeks (*n* = 12)**	**1 month (*n* = 14)**	**2–3 months (*n* = 14)**
IGF-I	12.8 (10.6–15.5)	63 (49–79)	56 (44–73)	66 (53–82)
IGF-II	64.4 (51.3–80.9)	67 (56–80)	60 (52–68)	64 (55–75)
F-IGF-I	0.10 (0.08–0.14)	29 (19–42)	25 (18–36)	31 (21–46)
IGFBP-1 RIA	1.5 (0.7–3.0)	250 (127–495)	273 (138–542)	273 (146–510)
IGFBP-2 RIA	29.6 (23.1–38.0)	53 (18–156)	69 (61–78)	66 (57–78)
IGFBP-2* WLB	5.1 (2.9–8.8)	80 (65–99)	68 (52–88)	57 (44–74)
IGFBP-3 RIA	94.6 (82.9–106.3)	74 (63–85)	69 (62–76)	71 (63–80)
IGFBP-3* WLB	75 (57–97)	84 (64–110)	61 (45–82)	69 (52–92)
IGFBP-3** PROT	0.35 (0.30–0.42)	–29 (–46 –5)***	–22 (–36 –6)***	–29 (–46 –7)***
IGFBP-4* WLB	3.7 (2.7–5.1)	146 (112–192)	140 (116–169)	150 (114–198)

*Arbitrary units. **Ratio of fragmented to total IGFBP-3. *n* = number of observations.
*** Percentage change from pretreatment levels.

parameters obtained in the different groups of patients are given as their geometric mean value with 95% confidence intervals of the mean, with the exception of IGFBP-3 protease activity where the arithmetic mean values are given. Considering statistical difference from pre-treatment levels, on-treatment values are significantly different when the confidence interval of the mean does not span the mean pretreatment value. *P* values were calculated using the Friedmans test (non-parametric analysis of variance).

RESULTS

9 patients obtained a partial or a complete response to treatment, 3 stable disease, while progressive disease was observed in 4 patients. Median time to progression was 18 weeks.

Alterations in the different IGF-parameters during treatment with diethylstilboestrol are shown in Table 2. The findings may be summarized as follows.

Treatment with diethylstilboestrol suppressed plasma IGF-I to a mean value of 56–66% of pretreatment levels ($P < 0.001$), IGF-II to 60–67% of pretreatment levels ($P < 0.001$) and free IGF-I to 25–31% of its pretreatment values ($P < 0.001$). Similarly, we observed a decrease in plasma IGFBP-2 to 53–69% of its pretreatment values as measured by RIA ($P = 0.001$), and 57–80% of pretreatment values when evaluated by Western ligand blot ($P = 0.0025$). We also observed a decrease in IGFBP-3 measured by RIA (69–74% of pretreatment values; $P = 0.001$), and Western ligand blot (61–84% of pretreatment values; $P = 0.07$), as well as a decrease in IGFBP-3 protease activity (mean decrease of 22–29%; $P = 0.03$). In contrast, plasma levels of IGFBP-1 measured by RIA and IGFBP-4 measured by Western ligand blot both increased (IGFBP-1 to 250–273% of pretreatment values; $P = 0.001$, and IGFBP-4 to 140–150% of pretreatment values; $P = 0.03$).

Previous treatment could potentially affect the results for patients number 2, 6 and 14. However, eliminating these patients had no influence on the results with the exception of IGFBP-3 protease activity, where the significant decrease in this parameter was lost.

Representative Western ligand blots and immunoblots are shown in Figure 1. For most parameters these alterations were fully developed after 2 weeks on treatment. Comparing patients obtaining a clinical response (CR or PR), stable disease, and progressive disease did not reveal any significant differences in the measured parameters between these patient groups.

DISCUSSION

Plasma levels of the different IGF-parameters measured before treatment were in the same range as previously reported by our group (Frost et al, 1996). Although some patients had short washout time, it is unlikely that previous treatment has significant impact on our results as most of these patients had received formestane, exemestane or anastrozole. Formestane (Frost et al, 1996) and exemestane (unpublished observations by our group)

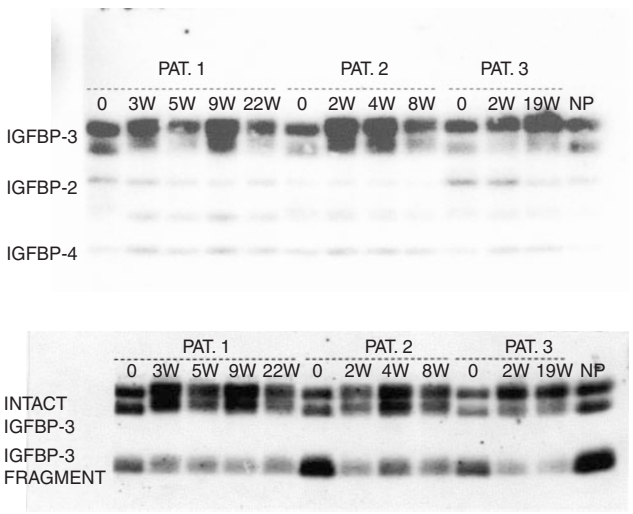

Figure 1 Western ligand blots (**A**) with corresponding immunoblots (**B**) from 3 patients before and during treatment with diethylstilboestrol. As these patients had a decrease in IGFBP-3 protease activity during treatment, alterations in intact IGFBP-3 do not reflect the fall in total 16FBP-3 observed in the whole treatment group

have been shown to have little effects on plasma IGF-I, while no data exists on anastrozole.

The moderate reduction in total IGF-I (30–40%) found in this study is in the range previously reported for oral oestrogens administered in doses regularly used for hormone replacement (Weissberger et al, 1991; Helle et al, 1996c), and during treatment with tamoxifen (Colletti et al, 1989; Helle et al, 1996b) or droloxifene (Helle et al, 1996a) in breast cancer patients. While these drugs also cause an increase in IGFBP-1 (Lønning et al, 1992; Helle et al, 1996a), the magnitude of this increase seems smaller with droloxifene, tamoxifen and hormone replacement therapy compared to that which we describe here. This may be related to drug doses for tamoxifen and droloxifene but also to the relative oestrogen agonistic versus antagonistic effects of these drugs. Tamoxifen has oestrogen agonistic effects on hepatic synthesis of sex hormone-binding globuline, thyroxin-binding globuline as well as lipoproteins (Fex et al, 1981; Love et al, 1991) and this drug is found to inhibit IGF-I gene expression in the liver (Huynh et al, 1993). Thus it is likely that the effects on plasma IGF-I levels (and possibly some IGFBPs) are due to oestrogen agonistic effects of diethylstilboestrol on hepatic synthesis.

Findings from in vitro studies are of limited value to explain the effects of diethylstilboestrol on plasma IGF-II and IGF-binding proteins. Oestradiol has been reported to increase expression of IGF-II (Lee et al, 1994), IGFBP-2 (Pratt and Pollak, 1993) and enhance the secretion of IGFBP-3 proteases (Salahifar et al, 2000) in breast cancer cell lines, while the opposite effects on plasma levels of these parameters are observed in this study.

The significant reduction in plasma IGF-II (30–40%) observed here, has not been recorded previously during therapy with oestrogens or anti-oestrogens. In contrast to IGF-I, IGF-II has not been considered a subject to regulation by endocrine drugs. The decrease in IGF-II observed in this study may be secondary to a decrease in IGFBP-3, which is also observed during treatment with oral oestrogens in replacement doses (Kam et al, 2000) as well as somatostatin analogues (Helle et al, 1998). However, in contrast to somatostatin analogues which decrease plasma growth hormone (GH) levels, the opposite effect is found during treatment with diethylstilboestrol (Bishop et al, 1985).

To our knowledge, free IGF-I has not been measured in previous studies evaluating the effect of endocrine treatment on the IGF-system. Here, we observed a drop in plasma levels of free IGF-I to a mean value of 25–31% of its pretreatment level. The decrease in free IGF-I may be somewhat more pronounced than expected based on the moderate decrease in total IGF-I. The observed decrease in IGFBP-3 protease activity may to some extent explain this finding, as the IGFBP-3 protease may facilitate release of IGF-I from the ternary complex (Lassarre and Binoux, 1994). The commercial IRMA kit used for measurement of free IGF-I in this study in general measures somewhat higher levels compared to what is recorded with use of ultrafiltration, probably due to inclusion of some easily dissociable IGF-I (Frystyk et al, 1999). However, the value most likely reflects the amount of IGF-I readily available to the tissues. It is also possible that increase in plasma levels of IGFBP-1 and IGFBP-4 may contribute to the reduced levels of free IGF-I. A greater suppression of free IGF-I, compared to total IGF-I, may indicate that free IGF-I measurement has more value as a surrogate marker for IGF-I bioavailability for breast cancer patients treated with endocrine therapy.

A moderate reduction in IGFBP-3 protease was observed. The patients included in the study in general had a low metastatic tumour burden and a slowly progressive disease. A high number of our patients responded to therapy with diethylstilboestrol, and we have recorded a decrease in IGFBP-3 protease activity for patients responding to tamoxifen (Helle et al, 1996b). A decrease in IGFBP-2 was also observed. Increased IGFBP-2 levels have been previously described for patients with prostate cancer (Cohen et al, 1993; Kanety et al, 1993). The mechanism behind the alterations in IGFBP-2 is not known, but there seems to be a strong positive correlation between IGFBP-3 protease activity and IGFBP-2 levels (unpublished observations). Thus, a decrease in IGFBP-2 may partly reflect a decrease in tumour burden during therapy.

The mechanisms by which oestrogens in high doses cause anti-tumour effects in breast cancer is unknown, but in vitro studies have shown a biphasic response curve for breast cancer cells with oestrogens in high concentrations being toxic to tumour cell growth (Lippman et al, 1976; Masamura et al, 1995). Also, there is evidence that oestrogens in high concentrations may induce apoptosis in vivo (Song et al, 2000). The toxicity threshold is lowered as an adaptive process during oestrogen deprivation (Masamura et al, 1995). Whether components of the IGF-system may be involved in these mechanisms is not known. The induction of the IGF-I receptor as well as the insulin receptor substrate-1 by oestrogens in breast cancer cell lines may indicate synergistic effects between these systems. (Stewart et al, 1990; Molloy et al, 2000). There is also evidence that IGF-I signalling may be necessary for maximal oestrogen receptor activation in some human breast cancer cell lines (Lee et al, 1997). The profound decrease in free IGF-I, and decrease in both IGF-I and -II, observed in this study, may be a part of the mechanism of action of this drug in breast cancer. However, further studies are needed to evaluate the complex interactions between oestrogens and the IGF-system in human breast cancer.

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