Pergamon

European Journal of Cancer, Vol. 34, No. 7, pp. 999–1003, 1998

Printed in Great Britain
0959-8049/98 $19.00+0.00

PII: S0959-8049(97)10170-8

Original Paper

Correlation Between Plasma Transforming Growth Factor-β1 and Second Primary Breast Cancer in a Chemoprevention Trial

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The relationship between plasma transforming growth factor-β1 (TGF-β1) and second primary breast cancer was explored in a prevention trial of the synthetic retinoid fenretinide (*N*-(4-hydroxyphenyl)retinamide; 4-HPR). Plasma concentrations of TGF-β1 were measured by radioimmunoassay in plasma obtained at randomisation and after approximately 1 year of intervention in 28 women treated with 4-HPR and 27 untreated controls with stage I breast cancer. Baseline and 1 year growth factor concentrations were not significantly different between treated and control groups. After a median follow-up of 65 months, an increase in TGF-β1 over 1 year was the only significant, independent predictor of a shorter survival free from secondary primary breast cancer. Moreover, the change in TGF-β1 levels had a tendency to influence the treatment effect on second breast cancer incidence. Our data suggest that the role of plasma TGF-β1 as a surrogate endpoint of breast carcinogenesis should be assessed further.

Key words: biomarkers, breast neoplasms, chemoprevention, intermediate endpoint, retinoids, transforming growth factor-β
Eur J Cancer, Vol. 34, No. 7, pp. 999–1003, 1998

INTRODUCTION

CHEMOPREVENTION AIMS at interfering with carcinogenesis at the preclinical cancer stage by using non-cytotoxic agents [1]. Since a major limitation of chemoprevention trials is the high cost ensuing from the large numbers of subjects and the prolonged period of observation that need to be studied, as well as the risk of detrimental effects [2], much emphasis has been given to the search for intermediate biomarkers which may reflect effective modulation of carcinogenesis by the study agent before the onset of the end-stage clinical event [3].

In 1987, our group started a phase III intervention trial of the synthetic all-*trans* retinoic acid derivative *N*-(4-hydroxyphenyl)retinamide (fenretinide or 4-HPR) in women with early breast cancer aged 30–70 years [4]. The primary endpoint is contralateral breast cancer, which currently provides the most suitable surrogate endpoint for breast cancer prevention in women who do not have an initial diagnosis of breast cancer. Indeed, the average incidence of contralateral breast cancer is 0.8% per year [5, 6], which is five to six times the age standardised incidence rate in the general population of northern Italy in the same age range [7]. Moreover, the rate of ipsilateral breast cancer is even higher, being two to three times the rate of contralateral breast cancer depending on the time since initial diagnosis [6].

In an attempt to find intermediate endpoints of 4-HPR modulation preceding the onset of second cancer, we implemented a number of ancillary studies. We focused our attention on blood concentrations of growth factors known to be relevant for breast carcinogenesis [8, 9] and potentially modulated by retinoids [10, 11], inasmuch as this simple approach has clear advantages compared with more invasive tissue sampling procedures. Recently, we demonstrated that 4-HPR modulates plasma concentration of insulin-like

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Received 29 Jul. 1997; revised 4 Nov. 1997; accepted 16 Dec. 1997.

growth factor-1 (IGF-1), with a significant interaction with menopausal status [12], with a decrease seen in premenopausal women as opposed to an increase in postmenopausal women, a pattern which seems to reflect the retinoid's preventative effect observed in an interim analysis [13].

In the present study, we measured the change in plasma concentrations of transforming growth factor-β1 (TGF-β1) in a subgroup of the same study. The human TGF-β family consists of multifunctional proteins which, once activated by release from the latent complex, are implicated in the regulation of diverse physiological processes, including cell proliferation, wound repair, angiogenesis and modulation of the immune system [14, 15]. Controversial data suggest that TGF-β has the potential either to inhibit [16, 17] or to promote [18–20] the progression of breast carcinogenesis. Moreover, recent work has provided evidence that TGF-β1 circulates at ng/ml concentrations in the plasma of normal subjects as well as of women with advanced breast cancer, suggesting a previously unsuspected endocrine role for this peptide [21–23].

PATIENTS AND METHODS

Subjects and treatment

We studied 55 breast cancer patients attending the unit of the National Cancer Institute of Genoa for semi-annual follow-up of a multicentre phase III secondary prevention trial involving 2,972 women [13]. This subgroup was selected based on the residual plasma availability within a cohort of 78 subjects who were consecutively recruited and studied for IGF-1 concentrations [12].

All patients were aged between 30 and 70 years, had stage I breast cancer (T_{1-2} N_0), had undergone radical mastectomy or quadrantectomy plus radiotherapy in the previous 10 years and had received no adjuvant systemic therapy. A detailed description of the inclusion criteria has been published elsewhere [4]. 28 women assigned to receive 4-HPR (RW Johnson Pharmaceutical Research Institute, Springhouse, Pennsylvania, U.S.A.), 200 mg orally daily for 5 years (two capsules at dinner) and 27 untreated controls formed the population on study. Subjects were accrued between December 1988 and November 1992. The study had received Institutional Review Board approval and written informed consent was obtained from each patient.

TGF-β1 levels were determined in plasma obtained at randomisation and during follow-up, ± standard error of the mean (SEM) interval of 11.4 ± 0.5 and 10.8 ± 0.4 months in the treated and control groups, respectively. Blood samples were obtained between 9 and 12 a.m., during clinical examination, using a syringe with a 19 G needle in order to minimise excessive platelet degranulation, although this was shown to be marginal in a previous study using the same drawing procedures [21]. Plasma aliquots obtained using sodium ethylene diamine tetra-acetic acid (EDTA) were separated by centrifugation and stored at − 70°C until the assay.

TGF-β1 assay

TGF-β1 levels were determined in duplicate in a single session by double-antibody radioimmunoassay using a non-equilibrium technique. Specific TGF-β1 antiserum, TGF-β1 obtained by DNA recombinant technology as a standard and ^{125}I-TGF-β1 were purchased from Du Pont de Nemours, NEN Division (Cologno Monzese, Italy). The sensitivity of this assay was 15 pg/tube. The intra-assay and interassay coefficients of variation were below 7 and 11.6%, respectively. According to the manufacturer, no cross-reactivity is found between TGF-β1 and TGF-β2 or -3 with the antibody used in this assay, up to concentrations of 50 ng/ml of peptides. Since the antibody recognises the active form of TGF-β1, a two step acidification/neutralisation activating process was required prior to the assay. Briefly, 100 µl samples were acidified by adding 10 µl of 0.5 M HCl 1.2 N, incubated for 15 min at 22°C, and then neutralised by adding 20 µl of 0.5 M HEPES, 0.72 M NaOH. All samples were diluted appropriately so as to reach a point on the curve where there was parallelism among recombinant TGF-β1, EDTA-plasma and serum (%B/Bo: 40–85).

Statistical methods

Differences in the median concentration of TGF-β1 between and within treatment groups were assessed by the Mann–Whitney *U* test and the Wilcoxon matched pairs rank test, respectively. The role of predictive factors on the level of plasma TGF-β1 after 1 year of treatment was evaluated by ordinary least squares multiple regression analysis [24]. To avoid the large right skewness in the distribution of the response variables, data were log-transformed. This leads to transformation from an additive regression model for the mean value to a multiplicative model for the median value. The resulting statistical index is referred to as the median ratio.

Backward non-automatic predictor selection was applied starting with interaction and main effect terms. Terms were removed from the models taking into account both their statistical significance and their contribution to the goodness-of-fit indices (leverage, residual and influence measures). In all models of 1 year log TGF-β1 concentrations, baseline log TGF-β1 concentrations were included to assess the change in growth factor levels during the treatment period.

Univariate analysis of survival free from second primary breast cancer by TGF-β1 levels over 1 year (positive versus negative Δ, where Δ is $\text{time}_1 - \text{time}_0$) was computed by the Kaplan–Meier method and statistically assessed by the log-rank test [25]. In this competing risk approach, subjects who developed distant metastasis ($n = 3$) were censored at the time of first event. Cox proportional hazards regression model was performed to assess the effect of each predictor on disease-free survival. To assess the effect of the change in TGF-β1 concentration on survival, both baseline and 1 year levels were used as a single internal continuous log-transformed time-dependent covariate [25]. Hence, the hazard ratio for TGF-β1 concentration represents a time-dependent relative risk index per unit increase in the covariate. The effects of TGF-β1 and 4-HPR were never removed from the models. Because of the limited number of events and the difficulty in distinguishing between local recurrence and second ipsilateral tumour, all new breast tumours, including those which arose from different quadrants of the operated breast ($n = 4$), were considered a second primary cancers and were pooled with contralateral breast cancers ($n = 3$).

RESULTS

Overall, there was a moderate correlation between baseline and 1 year TGF-β1 concentrations ($r = 0.29$, $P = 0.03$). None of the factors listed in Table 1 significantly contributed to explain baseline TGF-β1 concentrations (data not shown).

Table 1. *Patients' characteristics*

Factors and levels	Control ($n=27$)	4-HPR ($n=28$)
Age at study entry (years)		
≤ 52	15	13
> 52	12	15
Menopausal status		
Pre	11	11
Post	16	17
Parity		
≤ 1	19	14
> 1	8	14
Months of lactation		
≤ 6	18	21
> 6	9	7
Family history		
No relative with breast cancer	17	21
≥ 1 relative with breast cancer	10	7
Body mass index		
≤ 23.3	13	15
> 23.3	14	13
Platelets count ($10^3/mm^3$)		
≤ 242	13	15
> 242	14	13
Months from surgery		
≤ 24	13	17
> 24	14	11

4-HPR, *N*-(4-hydroxyphenyl)retinamide (or fenretinide).

At baseline, TGF-β1 levels were not significantly different between the treated group and the control group (median 6.1 ng/ml (range 2.0–20.3) versus 4.6 ng/ml (range 1.5–24.3), respectively). After 1 year of treatment, the median Δ TGF-β1 was − 1.6 (range − 11.9–50.8) in the treated group versus − 0.5 (range − 20.7–5.0) in the control group (not significant).

The effect of 4-HPR treatment and other predictors on TGF-β1 levels during the 1 year intervention period is shown in Table 2. A higher number of offspring (> 1) and a shorter period of lactation (< 6 months) were associated with

Table 2. *Multiple log-normal regression analysis of transforming growth factor*-β1 (TGF-β1) *levels after* 1 *year of intervention*

Factors and levels	Median ratio	95% CI	*P* value
Parity			< 0.001
≤ 1	1.0	(Ref.)	
> 1	2.5	1.5–4.0	
Months of lactation			0.008
≤ 6	1.0	(Ref.)	
> 6	0.5	0.3–0.8	
Treatment by family history of breast cancer			< 0.01
Control			
No relative with breast cancer	1.0	(Ref.)	
≥ 1 relative with breast cancer	1.5	0.9–2.4	
4-HPR			
No relative with breast cancer	1.0	(Ref.)	
≥ 1 relative with breast cancer	0.5	0.3–0.9	

Baseline log TGF-β1 level is included in the model (F test = 6.6; P value = 0.01). Overall F test = 5.8; P value < 0.001; $R^2 = 0.50$. 95% CI, 95% confidence interval for median ratio adjusted by linear and squared age; P value, significance level of F test; Ref., reference category; 4-HPR, *N*-(4-hydroxyphenyl)retinamide (or fenretinide).

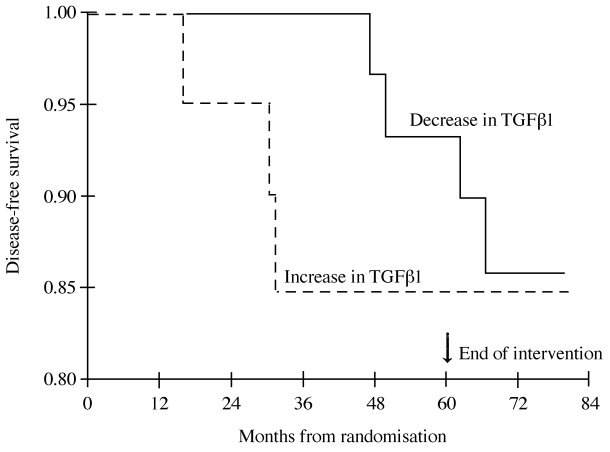

Figure 1. Effect of the transforming growth factor-β1 change in plasma (TGF-β1) over 1 year on survival free from second primary cancer (not significant).

increased TGF-β1 levels. In addition, TGF-β1 levels were affected by a significant interaction between 4-HPR treatment and family history of breast cancer, in that the administration of the retinoid induced a significant decrease in TGF-β1 concentrations in women with a positive family history in contrast to an increase observed in the same subgroup of control women.

There were four events in the 4-HPR group at 16, 30, 30 and 49 months and three events in the control group at 47, 52 and 67 months, respectively. When the TGF-β1 data were analysed according to subsequent events, the median baseline growth factor concentration was 4.9 ng/ml (range 2.1–14.1) in women who subsequently developed a second breast cancer compared with 6.1 ng/ml (range 1.5–24.3) in those who did not (not significant). Univariate analysis of the change in TGF-β1 (positive Δ versus negative Δ) on disease-free survival is represented in Figure 1. After a median follow-up time of 65 months (range 16–82), there was no significant difference in survival free from second primary breast cancer according to the change in TGF-β1 levels (log-rank test = 0.13, $P = 0.38$). However, multivariate regression analysis showed that the change in TGF-β1 over 1 year was the

Table 3. *Multivariate analysis of survival free from second primary breast cancer*

Model	Covariate	Hazard ratio	95% CI	*P* value
1*	Treatment			0.420
	Control	1.00	(Ref.)	
	4-HPR	0.46	0.07–3.12	
	Log (TGF-β1)†	2.76‡	1.13–7.67	0.037
2	Log (TGF-β1)†	2.23‡	0.99–5.01	0.054
3	Treatment			0.970
	Control	1.00	(Ref.)	
	4-HPR	1.04	0.23–4.77	

Hazard ratio adjusted for age, body mass index and years since surgery, 95% CI, 95% confidence interval for hazard ratio; P value: significance level of likelihood ratio test; Ref., reference category; 4-HPR, *N*-(4-hydroxyphenyl)retinamide (or fenretinide); TGF-β1, transforming growth factor-β1.

*Overall likelihood ratio test = 10.1; P value = 0.07. †Time-dependent covariate. ‡Hazard ratio per unit increase in log (TGF-β1).

only significant, independent predictor of disease-free survival (Table 3). Three separate models are presented. In the first, where both treatment and TGF-β1 change are included, an increase in TGF-β1 concentrations was associated with a shorter survival free from second primary breast cancer, while treatment exerted a non-significant protective effect. Interestingly, while treatment removal from the model did not substantially affect the detrimental effect of TGF-β1 increase (model 2), TGF-β1 removal led to the loss of the 4-HPR effect (model 3).

DISCUSSION

Our data show that an increase in TGF-β1 concentration over 1 year is a significant predictor of shorter survival free from second primary breast cancer in a group of breast cancer patients participating in a prevention trial of the synthetic retinoid 4-HPR. The effect of TGF-β1 levels on breast cancer was only marginally dependent on 4-HPR, inasmuch as the predictive effect of TGF-β1 was not affected by the removal of treatment from the Cox model. In contrast, the change in TGF-β1 had a tendency to influence the intervention effect, suggesting a partial involvement of the growth factor in the modulation of breast carcinogenesis by the retinoid [26]. Indeed, several lines of evidence point to an interaction between the steroid/retinoid receptor superfamily and TGF-β1 pathways in the regulation of cell homeostasis [11]. Although the effect of 4-HPR *per se* tended to be beneficial, the limited power of the study does not allow any reliable interpretation of treatment results. Moreover, an interim analysis of the phase III trial of 4-HPR shows a complex pattern of activity of this compound on contralateral breast cancer, with an interaction with menopausal status (or age) which apparently modifies its preventive effects [13].

The pattern of TGF-β1 modulation was likewise complex, involving a significant modification of the 4-HPR effect according to a family history of breast cancer, in addition to the confounding effect of parity and lactation. The finding that 4-HPR intervention tends to reduce TGF-β1 levels in women with a family history of breast cancer might suggest potential genetic interactions between the two superfamilies. Moreover, reproductive factors such as parity and lactation influenced TGF-β1 levels, further supporting the contention of a regulation by hormonal factors [17, 27]. At present, the reasons for these findings are still unclear, but clearly warrant further investigation.

There are several limitations to our study. The limited power of the study, which accounts for the uncertainty in risk estimates, defines the exploratory nature of our study, which may help to generate hypotheses to be evaluated in subsequent *ad hoc* studies. Moreover, the study was not specifically designed to address this issue, so that the risk of a selection bias cannot be excluded. Finally, the current inability of the assays to distinguish between the latent and the active form of TGF-β1 does not allow insight into the functional meaning of our findings.

Our results seems to be consistent with the vast majority of *in vivo* studies on TGF-β1 modulation of breast carcinogenesis, where the growth factor showed promoting effects [23, 28]. Several mechanisms underlying these effects have been advocated, including inhibition of both humoral and cellular immunity, promotion of angiogenesis and synthesis and deposition of extracellular matrix, with consequent increased cell adhesiveness and metastasis formation (reviewed in [18]). It has been suggested that the growth factor may have preventative effects in early phases, as opposed to promoting effects in late phases of breast carcinogenesis [25], consistent with the evidence that the growth factor has biphasic effects during multistage carcinogenesis [29].

Conflicting results have also been reported on the causal relationship between plasma TGF-β1 concentrations and breast cancer. In their initial study, Anscher and associates [21] reported no apparent relationship between tumour burden and plasma TGF-β1 levels in 41 breast cancer women following induction chemotherapy. In contrast, the same group has recently shown, in a series of 26 women, that high concentrations of plasma TGF-β1 (> 10 ng/ml) correlated with the presence and the extent of primary breast cancer and were reduced after tumour excision in most cases [23]. This would suggest that an elevation in plasma TGF-β1 is an early marker for breast cancer. Conversely, Wakefield and colleagues [22] showed no evidence of increased TGF-β1 levels in 26 of 28 women with advanced breast cancer. While our results seem to support the former conclusion, additional studies are clearly needed, particularly taking into account the short half-life of TGF-β1 [30].

In conclusion, an increase in plasma TGF-β1 concentration over 1 year was associated with a shorter survival free from second primary breast cancer in women with early breast cancer. Since the growth factor might partially be implicated in the modulation of breast carcinogenesis by 4-HPR, its role as a surrogate endpoint of breast carcinogenesis should be assessed further.

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Acknowledgements—Supported by NIH/NCI grants UO1 CA-38567 and CA-56457 and by the Italian Association for Cancer Research (AIRC). AD and AC work on a chemoprevention programme supported by the Italian Foundation for Cancer Research (FIRC). The authors are indebted to Dr Aliana Guerrieri Gonzaga, Dr Tom Wiley and Miss Alessia Fossati for their skillful technical assistance.