Changes in Messenger RNA Expression of Protein Kinase A Regulatory Subunit Iα in Breast Cancer Patients Treated with Tamoxifen

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ABSTRACT

RNA for protein kinase A regulatory subunit Iα (RIα) has been measured in tumors from 32 breast cancer patients before and during primary treatment with tamoxifen. Values in pretreatment specimens were significantly higher in tumors subsequently responding to treatment as compared with those not (P = 0.004 by Mann-Whitney U test). Furthermore, whereas levels fell with treatment in 16 of the 24 responding tumors, they did not in any of the 8 tamoxifen-resistant tumors (and indeed rose in 6 cases). These results suggest that measurement of RIα mRNA may help in identifying endocrine-dependent breast cancers and provide further evidence of the involvement of the protein kinase A system in response and resistance to tamoxifen treatment.

INTRODUCTION

Tamoxifen is a nonsteroidal antiestrogen widely used to treat hormone-dependent breast cancer (1). The drug has established efficacy in patients with advanced disease (1–3), and meta-analysis has confirmed improved survival in patients with early breast cancer treated adjuvantly (4). Despite this, the mechanisms by which tamoxifen achieves its antitumor action are incompletely understood. Major effects appear to be mediated through the ER2 (5), with benefits being primarily seen in patients with ER-positive cancers and receptor-negative tumors rarely responding (4, 6–8). However, even in ER-positive disease, response rates rarely exceed 60%, and many patients ultimately relapse on therapy (9–11). There is thus an immediate need to identify factors that predict for response and determine both primary and acquired resistance. Lack of sensitivity to endocrine therapy is likely to be multifactorial and may include compensatory hormonal mechanisms (12), altered drug pharmacology (13, 14), efflux pumps (15), outgrowth of hormone-independent clones of cells (16), and the constitutive production of other growth factors and mitogens (17). However, more recently, attention has focused on the possibility that other signaling pathways may impact on the transcriptional activities of steroid hormones and their regulation by antihormones (18). For example, it has been shown that phosphorylation of steroid receptors mediated by the PKA system may, in part, bypass the need for ligand activation (19, 20). In this respect, it is relevant that we have shown that tumor levels of regulatory subunits of PKA in combination with ERs may better predict response to endocrine therapy than ERs alone (21). The regulatory subunits (R) of PKA fall into two major classes, RI and RII (22). RI seems to be overexpressed in cancer cells and may program for proliferation and aggressive behavior (23). Overexpression of RII may also be associated with the acquisition of drug resistance (24). We have recently developed a quantitative RT-PCR methodology by which to measure mRNA for RIα (25), and it was of interest to determine the effects of tamoxifen on RIα expression in breast cancers. To do this, a cohort of elderly patients with breast cancer given primary systemic therapy with tamoxifen has been studied before treatment and at definitive surgery. In this way, it has been possible to measure RIα mRNA in individual cancers before and during tamoxifen treatment and relate expression to clinical response in the same tumors (as monitored by serial ultrasound of the breast).

MATERIALS AND METHODS

Patients. Thirty-two patients ages 70 years or older presenting to the Edinburgh Breast Unit were entered into the study. All had histologically confirmed breast cancer and tumors with ER levels >10 fmol/mg cytosol protein. The intent was to treat all patients with primary systemic therapy comprising tamoxifen (20 mg/day) for 3 months. However, in two patients, tamoxifen was discontinued after 1 month because of evidence of increased tumor size, and 15 patients elected to continue treatment beyond 3 months (up to a maximum of 10 months). During treatment, tumor size was monitored monthly by ultrasound and caliper measurements. All patients had an initial wedge biopsy of their tumor before treatment (for histological confirmation of malignancy and ER assay) and definitive surgery (either wide local excision or mastectomy) to remove residual tumor at the end of the study period. Tumor material was stored in liquid nitrogen until extraction of RNA.

Extraction of Tumor RNA. Approximately 200 mg of each sample were pulverized in a tissue dismembranator (Braun, Melsungen, Germany), and total RNA was extracted by the lithium chloride method of Auffrey and Rougeon (26) as modified by MacCallum et al. (27). Resultant RNA (approximately
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Quantitative RT-PCR. Rlα mRNA was measured by a quantitative RT-PCR method using plasmid-derived, mutated Rlα as an internal standard. Full experimental details of this method and validation have been described previously (25). Briefly, 1 μg of total RNA for each sample was reverse transcribed with 100 ng of random hexamer oligonucleotide as a primer at 42°C for 1 h in a thermal cycler (Techne PHC). The resultant cDNA was subsequently stored at −20°C before PCR.

The PCR internal standard was constructed using site-directed mutagenic primers and mix-melt PCR. The PCR product was modified to include an EcoRV restriction enzyme site. This mutant used the same primers as the target mRNA and differed in sequence by just 4 bases. This resulted in a <0.5% change in subsequent labeling with [32P]dCTP in a 430-bp product. PCR was carried out using known log dilutions of mutant cloned plasmid (100, 10, 1, and 0.1 pg) and equal amounts of unknown cDNA. In addition, normal cloned plasmid quality control samples of known concentration were included in each assay to monitor assay variations.

PCR was carried out over 26 cycles in a 100-μl volume containing 1.25 mM deoxynucleotide triphosphates, 1.25 mM MgCl₂, and 0.5 units of Taq polymerase (Imperial Cancer Research Fund, Oxford) in the presence of 3.7 kBq [32P]dCTP (Amersham International, Little Chalfont, UK). A 20-μl aliquot of each PCR product was digested with restriction enzyme (5–10 units of EcoRV, Promega, Southampton, UK). Labeled digests were separated on 6% polyacrylamide gels for 2 h at 35 mA per gel, dried, and exposed to preflashed X-omat AR film fitted with intensifier screens overnight at −80°C. Bands corresponding to the mutant and normal arms of the PCR products aligned with radioactive ink as marker were excised, and the point of equal counts (crossover) between each sample set and plasmid mutant standard allowed the estimate of its concentration.

To control for cellularity changes in pre- and posttreatment biopsies, a noncompetitive PCR was carried out on each sample using primers that target a 555-bp sequence on the GAPDH "housekeeping" gene.

RESULTS

Clinical Response to Tamoxifen Treatment. Response to tamoxifen as monitored by sequential ultrasound measurements of the breast was defined as >20% reduction in tumor size between pre- and posttreatment biopsies (28). On this basis, 24 patients were classified as responders, experiencing tumor reduction in size of between 21 and 96%; in the remaining 8 patients, tumor size either remained similar (6 cases) or increased in size (2 patients; 10–25% increase in size within 1 month of treatment). Levels of ERs were significantly higher in responding tumors (median, 363 fmol/mg protein; range, 72–1496) as compared with nonresponding tumors (median, 57 fmol/mg protein; range, 10–529), although, as can be seen from the ranges, there was an overlap in values between the groups.

Measurements of Rlα mRNA. A typical example of an autoradiogram of a quantitative RT-PCR from the primary cancers of two patients taken both before and during treatment with tamoxifen is shown in Fig. 1. This illustrates variable crossover points in different tumor specimens between sample mRNA and the cut product of mutant Rlα mRNA added as internal standard. Each sample of the 32 tumors contained detectable amounts of Rlα mRNA as assayed using this methodology, but levels varied greatly between different specimens, ranging from 0.1 to 74 pg. These were not associated with values for ERs.

Levels of mRNA were not significantly different between samples of the same tumor taken before and during treatment with tamoxifen, as determined by paired analysis of the total patient population. However, subdivision of the tumors according to clinical response revealed interesting trends. Thus, as is shown in Fig. 2, the pretreatment level of mRNA was significantly higher in cancers that subsequently responded to tamoxifen as compared with non-responding tumors (P = 0.004 by Mann Whitney U test); this difference was not apparent in tamoxifen-treated tissue taken from the same tumors. Fig. 2 also illustrates the change in Rlα mRNA associated with treatment in individual tumors. Although levels of mRNA decreased in 18 of
the 24 responding tumors ($P = 0.004$ by Wilcoxon signed pairs test), it did not decrease in any of the nonresponding cancers; indeed, a rise was seen in 6 of the 8 cancers ($P = 0.03$ by Wilcoxon signed pairs test). Given that we have calculated previously that intra- and interassay variation may cause up to 2-fold differences in values (25), it was of interest to subdivide tumors according to changes that exceed this variation. These results are summarized in Table 1, which shows that, whereas the majority of responding tumors showed a substantial decrease in mRNA with treatment, most of the nonresponders displayed an increase. These differences between responding and nonresponding tumors were statistically significant ($P = 0.0001$ by $\chi^2$ test). A similar trend was seen when using a 5-fold difference between treatment and on-treatment material to classify into either a decrease or an increase in RNA (Table 1). It was also of interest to compare the quantitative change in RIA mRNA with the degree of tumor response. This is shown in Fig. 3, which illustrates a highly significant inverse correlation between the ratio of mRNA in tumor samples taken before:mRNA taken during treatment and the change in tumor volume occurring with treatment ($P = 0.0003$ by Spearman’s rank test).

**DISCUSSION**

We have recently developed and validated a RT-PCR method for the quantitation of RIA mRNA (25). The present article describes the use of this assay to measure RIA mRNA in tumor samples taken sequentially before and during treatment with tamoxifen of patients with large primary breast cancers. Not surprisingly, because RIA seems to be ubiquitous, RNA for RIA was detected in specimens from all tumors. However, levels varied greatly both between different tumors and within the same tumor studied before and during treatment. Because the direction of change with treatment appeared random, no significant difference was found between pretreatment and tamoxifen-treated specimens when the total population of cancers was investigated.

However, the particular attribute of the present study is, that by monitoring the size of the primary tumor, clinical response to treatment can be determined in individual patients. Interestingly, therefore, subdivision of tumors according to clinical response revealed some statistically significant trends, including the following: (a) tumors subsequently responding to tamoxifen had higher RIA mRNA levels in pretreatment biopsies than did nonresponding cancers; (b) tamoxifen treatment of responding tumors tended to be associated with a decrease in mRNA levels for RIA, whereas values in nonresponding tumors remained similar or increased; and (c) the degree of change with treatment was correlated with magnitude of clinical response. These effects were not nonspecific, in that semiquantitative RT-PCR of the housekeeping gene GAPDH did not reveal parallel changes. Such observations suggest that levels and changes of RIA mRNA are associated with endocrine responsiveness. It is therefore worth considering the reasons for this and the implications of the findings.

We have reported previously that measurements of regulatory subunits of PKA as measured by cyclic AMP binding within tumor cytosols may help predict the endocrine responsiveness of ER-positive breast cancers, relative overexpression being associated with resistant tumors (21). This is apparently at odds with the present finding, which suggests that higher tumor levels of RIA mRNA are more likely to confer responsiveness to tamoxifen treatment. There are, however, major differences
between our two studies, including (a) the component of the PKA system measured, (b) the nature of the tumor specimens, (c) the patient population, and (d) the type of endocrine therapy. These parameters are worthy of further consideration. Our early study measured total cyclic AMP binding, which will reflect protein levels of both RI and RII, whereas the present investigation assessed only RIA at the level of mRNA. Although we have shown that RIA is the major component of the total binding in breast cancer cytosols (29), levels of mRNA and protein may not necessarily be directly correlated. Indeed, there is evidence that protein levels of regulatory subunits may be equally affected by posttranslational stability factors (30) as those controlling transcription (31, 32). Clearly, there is an immediate need to address this issue, and it would have been helpful to have measured both protein and mRNA in the present study, but amounts of tissue were limited, and we currently do not have a quantitative assay for RIA protein. Additionally, our earlier investigations assayed a mixture of tumor specimens (including primary and metastatic disease) from patients with advanced breast cancer treated with a spectrum of different endocrine therapies; this contrasts to the present study, which exclusively assayed primary tumors from patients with "early" disease treated by tamoxifen alone. Although there is no a priori reason to believe that levels of RIA mRNA differ between primary and metastatic lesions or with advancement of disease, it may be that the PKA system is more influential in modulating response to antihormones (this is considered in more detail below).

It is possible that the high levels of RIA mRNA in certain hormone-dependent tumors may have resulted from estrogen stimulation. This could occur directly or via an indirect growth response. Thus, estrogen is capable of increasing cyclic AMP levels in breast cancer cell lines (33, 34); this in turn could induce the expression of RI (35). Similarly, estrogen has been shown to increase RI expression in the endometrium during the proliferative phase of the menstrual cycle (36) and in hormone-sensitive tissues when given as replacement therapy in castrated rodents (37). Equally, increased RI expression has been associated with dividing cells (23, 38–40), and it may be that, if estrogen stimulates cell growth, high RI mRNA may simply reflect a state of high proliferation. In this case, the antiproliferative effects of tamoxifen might be expected to reduce RI expression, a consequence that would be compatible with the findings of the present study. This would also be consistent with the decrease in concentration of RI subunits that accompanies castration-induced atrophy of hormone-dependent tissues (37, 41). If RI expression is associated with tumor growth rates, an important consideration is whether changes in RIA precede and program for changes in proliferation or are a consequence of it. Results from the present study cannot address this issue, because the tamoxifen-treated specimens from responsive tumors were taken only after clear evidence of regression. However, the close quantitative correlation between the fall in RIA mRNA and the reduction in tumor size would be consistent with a decreased population of proliferating cells, although changes in RIA mRNA and proliferation as measured by KiS, were often discordant (data not shown). An important extension of the studies would be to obtain additional tumor samples at an early time point of treatment preceding clinical response. It would also be of value to know whether parallel changes also occur in other regulatory units, such as those that occur in other hormone-dependent tissue following castration (37) or whether compensatory changes occur in RII mRNA relative to RI, as has been reported at the level of protein in cancer cell lines (32, 42).

A further interesting finding was the observation that the RIA mRNA not only did not decrease following tamoxifen treatment of nonresponsive tumors but often increased. This may reflect the outgrowth of high proliferative (and therefore high RI-expressing) hormone-insensitive cells. Equally, it may be that the increase is associated with the process of resistance. There is increasing evidence that high kinase activity (including PKA; Refs. 20, 43, and 44), through hyperphosphorylation of either the ER (44, 45) or accessory proteins (46), may not only induce a hormone-resistant phenotype (43–46), but it may change the pharmacology of tamoxifen from a partial estrogen agonist to one with full agonist activity (19, 46, 47). Interestingly, abnormal expression of RI may also be associated with other forms of resistance (19, 24, 47–50). Thus, strategies to reduce RI expression can reverse the multidrug-resistant phenotype (24, 48–50). In this respect, it is relevant that we have
shown that tamoxifen may induce P-glycoprotein expression in a cohort of hormone-insensitive tumors (28). The possibility therefore exists that antitumor resistance can be circumvented by the use of agents that either reduce R(3) expression or block its increase, e.g., site-selected cAMP analogues and antisense technology.

In summary, although the present observations are based on a relatively small number of tumors, the findings suggest that measurements of R(3) mRNA may aid in predicting response of ER-positive tumors to tamoxifen and may also help elucidate mechanisms by which breast cancers may respond or become resistant to endocrine therapy. More extensive investigations are thus warranted.

REFERENCES

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