Cyclin D1 Overexpression Sensitizes Breast Cancer Cells to Fenretinide

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ABSTRACT

Purpose: Fenretinide has shown promise in the chemoprevention of breast cancer, a tumor type in which the oncogene cyclin D1 is overexpressed frequently. We aimed at determining the effect of cyclin D1 level on the response to fenretinide treatment.

Experimental Design: Stable clones of T47-D cells were created to overexpress cyclin D1 or a mutant of cyclin D1, injected in nude mice for xenograft formation, and the rate of tumor growth and tumor regression determined.

Results: We show here that cells overexpressing cyclin D1 are significantly more sensitive to fenretinide than genetically matched cells that express low levels of cyclin D1, and that fenretinide prevents tumor formation arising from cyclin D1-overexpressing cells. Furthermore, we show that fenretinide is also able to promote the regression of cyclin D1-positive tumors. We also show that cells expressing a mutant of cyclin D1 that cannot bind to cdk4 are also more sensitive to fenretinide.

Conclusions: These results suggest that fenretinide may be particularly useful in the treatment of cyclin D1-positive breast cancers, and that the interaction between cyclin D1 and fenretinide is independent of cyclin D1 binding to cdk4.

INTRODUCTION

One of the biggest challenges in oncology is to predict the individual cancer patient response to a particular treatment. Ultimately, establishing a correlation between the expression of one gene and response to a particular drug would be ideal. One stunning example of such a correlation is between the presence of the PML-RARα fusion protein in acute promyelocytic leukemia and treatment using ATRA3 (1). ATRA has been shown recently to also accelerate the degradation of cyclin D1 (2). Cdks are key cell cycle regulators, and their activities are modulated at several levels, including the binding to their regulatory subunits, the cyclins (3). Among the cyclin subunits, α-type cyclins associate with cdk4 and 6 to phosphorylate the Rb protein. Hyperphosphorylation of Rb promotes the release of the E2F family of transcription factors that then promotes entry into S phase through activation of key target genes (4). In human breast cancer, abnormal accumulation of cyclin D1 is observed in up to 35% of cases, and overexpression of cyclin D1 in mouse mammary glands leads to carcinoma (5, 6). Cyclin D1 is overexpressed by several mechanisms (7–9) and was shown recently to be essential for tumor formation in MMTV-erb-2 transgenic mice (10). The particular importance of cyclin D1 in breast cancer is additionally supported by the observation that cyclin D1 knockout mice show a defect in alveolar proliferation during pregnancy (11, 12).

Overexpression of cyclin D1 has been reported to promote sensitivity to retinoic acid in vitro (13). This result raises the interesting possibility that retinoic acid may be useful for the treatment of cyclin D1-overexpressing tumors. However, this study did not address the efficacy of ATRA against cyclin D1-overexpressing tumors in vivo and, furthermore, the use of ATRA against breast cancer is limited by its toxicity. Fenretinide is a synthetic analogue of ATRA, with several advantages over ATRA for the treatment of solid cancers including a low toxicity profile, a stable plasma concentration, and the ability to concentrate in the breast (14). Unlike ATRA, fenretinide induces apoptosis (15) and is a poor inducer of differentiation (16). The results of a clinical trial testing the efficacy of fenretinide in preventing the recurrence of breast cancer in early stage patients who had their primary removed surgically were reported recently. This trial indicated a potential benefit of fenretinide treatment in premenopausal women (17). However, because this trial was not targeted at a specific molecular marker, the optimal efficacy of fenretinide may not have been fully revealed. We initiated this study to test the effect of cyclin D1 overexpression on the response to fenretinide treatment in a xenograft model. We found that cyclin D1 overexpression sensitizes breast cancer cells to killing by fenretinide in vitro and in vivo. Our results raise the exciting possibility that fenretinide may be of particular benefit to the treatment of breast cancers characterized by the overexpression of cyclin D1.

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3 The abbreviations used are: ATRA, all-trans retinoic acid; cdk, cyclin-dependent kinase; Rb, retinoblastoma; MMTV, mouse mammary tumor virus; C, control; P, parental; F, fenretinide; D, D1; CI, confidence interval.
MATERIALS AND METHODS

Cell Culture, Plasmids, and Transfections. Rb-positive cell lines T47-D and MCF-7 were grown in RPMI 1640 or DMEM, respectively, supplemented with 10% FCS, insulin (100 IU/ml), hydrocortisone (0.5 mg/ml), and antibiotics (Life Technologies, Inc.). cDNA encoding the coding sequence of cyclin D1-HA was cloned in the pCDNA3 expression vector (Invitrogen).

Transfections of cyclin D1-HA-pcDNA3 plasmid into T47-D cells was performed using the FuGENE 6 system, as described by the manufacturer (Boehringer Mannheim). Clones stably expressing cyclin D1-hemagglutinin or empty vector were selected using G418 at a final concentration of 0.78 mg/ml.

Western Analysis. Protein extracts and cyclin D1 Western analyses were performed as described previously (8).

Animals and Xenograft Implantation. Eight-week-old BALB/c nude mice were purchased from the Animal Research Center, (Perth, Australia). Before injection of T47-D and D1 cells, a 17β-estradiol pellet (0.72 mg/ml) was inserted s.c. in the upper back of each mouse. To insert the estradiol pellet, mice were anesthetized using methoxyflurane. A small incision was made and the pellet inserted using precision trochar. The incision was sealed using an auto clip.

Before the s.c. injection, 5 × 10^6 cell pellets were mixed with an equal volume of Matrigel (Basement Membrane Matrix; Becton Dickinson). The mixture was injected s.c. in the lower back of each animal using a 26-gauge needle.

Fenretinide and Control Treatment. One-hundred mg capsules containing fenretinide in 500 μl of corn oil were obtained from the National Cancer Institute. The corn oil containing fenretinide was extracted from the gelatin capsule using a 1-ml syringe and mixed with 500 μl of cremophor. The mixture was than diluted in PBS at a final concentration of 1.8 μg/ml and the stock solution protected from light. One-hundred μl of fenretinide stock solution was administered to mice p.o. by gavage using a 1-ml syringe, thereby providing a dose of 120 mg/kg of body weight. Mock-treated mice received by gavage using a 1-ml syringe, thereby providing a dose of 120 mg/kg of body weight. One-hundred μl of fenretinide stock solution was administered to mice p.o. by gavage using a 1-ml syringe, thereby providing a dose of 120 mg/kg of body weight. Mock-treated mice received by gavage using a 1-ml syringe, thereby providing a dose of 120 mg/kg of body weight.

Measurement of Tumor Size. Tumors were measured weekly using a digital caliper (mm). Two independent measurements were taken for each tumor and their resulting average used to obtain tumor volume.

Statistical Methods. To examine the effects of fenretinide treatment on the growth of parental and D1 cells in vitro, parental and D1 cells were plated and treated with five different levels of fenretinide concentration. The percentage of growth inhibition was recorded for each concentration. The experiment was repeated twice resulting in three observations for each cell line and each concentration level. Results from all three of the experiments were very similar. The equality of the growth inhibition rates corresponding to the parental and D1 cells over the range of fenretinide concentrations studied was tested using an analysis of covariance. The P obtained was a result of a comparison of the equality of slopes for the two cell lines.

In the tumor prevention and regression studies, all of the formal analyses were performed on the logarithms of the measured volumes (Y) according to the relationship, Y = 100 × log_e (X) as tumor growth was expected to be exponential. These Y values are referred to as log volumes. The slope of a regression of the log volume on time for each mouse was used as a summary measure over the period studied. The slope represents the percentage of increase in tumor volume per week for each mouse and is also referred to as the tumor growth rate. Comparisons between two given groups of mice were based on comparing these slopes. The two-sample t test was used for each comparison. The nonparametric Mann-Whitney test was also applied to confirm the results. The Mann-Whitney test results were only reported when both t test and Mann-Whitney tests resulted in different conclusions. In the tumor formation study, the slope was calculated using only data from weeks 2–9, because tumor shrinkage between week 1 and week 2 occurred in the majority of mice, which was normal and independent of tumor growth. Three mice in the tumor formation study were excluded from the analyses, as they were sacrificed before week 7 because of ill-health (1 in parental-control, 1 in D1-fenretinide, and 1 in parental-fenretinide). In analyzing the tumor growth rate from week 1 to 5 in the tumor regression study, two mice were included, as they were sacrificed before week 5 because of ill-health (both in D1-control) and similarly, an additional two mice were excluded in the analysis from week 5 to 11, as they were sacrificed before week 8 because of the same reason (1 in parental-control and 1 in parental-fenretinide).

All of the statistical tests were two-sided, and a P < 0.05 was considered to be statistically significant. Graphs were plotted using EXCEL 2000, and statistical analyses were performed using SPSS version 11.0.1 for Windows.

RESULTS

Cyclin D1 Overexpression Lead to Increased Sensitivity to Fenretinide in Vitro. Overexpression of cyclin D1 promotes sensitivity to ATRA in breast cancer cells (13). However, ATRA has shown clinical limitation in the treatment of breast cancer, we turned our attention to the synthetic analogue of ATRA, fenretinide, which shows promising results in recent clinical trials in the prevention of breast cancer (17). To test the effects of cyclin D1 overexpression on fenretinide treatment, we established T47-D-based stable clones overexpressing constitutively an HA-tagged version of cyclin D1 under the control of the cytomegalovirus promoter. The level of cyclin D1-HA expression in three representative clones is shown in Fig. 1A. To avoid the possibility of indirect effects because of the site of integration of the cyclin D1 gene in each clone, the three clones were pooled and the mixed cell population termed D1 cells. Parental cells refer to a pool of T47-D clones that were transfected with the cytomegalovirus vector alone.

The effect of fenretinide treatment on the growth of parental (T-47D) and D1 cells was examined. Over the range of concentrations studied, the percentage of growth rate inhibition of D1 cells was higher than that of parental cells (Fig. 1B; P < 0.0005). When exposed to 5 μM fenretinide, the growth of D1 cells was inhibited by 57% compared with 47% inhibition of parental cells. A similar result was obtained when cyclin D1 was overexpressed using a cell line where cyclin D1 is under the control of a zinc-inducible promoter (data not shown). This
were determined by Western analysis using an anticyclin D1 antibody. Parental and cyclin D1-HA independent clones of T-47D cells stably expressing cyclin D1-HA were harvested and protein extracted. Levels of cyclin D1 and cyclin D1-HA in vitro response to Fenretinide treatment (Fig. 1A), overexpression of cyclin D1 promotes growth inhibition in parental and D1 cells (Fig. 1C) supporting the previous finding that fenretinide affects cyclin D1 transcription (18). Interestingly, exogenous cyclin D1-HA levels were also reduced by fenretinide, although the effect was modest, raising the possibility that fenretinide may also affect to some extent cyclin D1 proteolysis. In agreement with previous observations (18), we found no specific effect on cell cycle arrest after fenretinide treatment (data not shown). However, we found that overexpression of cyclin D1 increased the level of fenretinide-mediated apoptosis (Fig. 1D). These results show that overexpression of cyclin D1 leads to increased apoptosis after fenretinide treatment compared with genetically identical cells that do not express cyclin D1 at such elevated levels.

**Fenretinide Treatment Prevents Growth of Cyclin D1-overexpressing Xenograft.** To determine whether overexpression of cyclin D1 sensitizes cells to fenretinide treatment in vivo, we next used parental and D1 cells for the formation of xenografted tumors in nude mice. Mice were divided in four groups. Mice in group 1 were injected with P cells and were mock treated, and we refer to this group as the PC group. Mice in group 2 were injected with P cells and received F treatment (PF group). Mice in group 3 were injected with D cells and received mock treatment (DC group). Finally, mice in group 4 were injected with D cells and were treated with F (DF group). Tumor formation was monitored by weekly size measurements over a period of 9 weeks. Fenretinide or control treatments were initiated 1 day after cells were injected s.c. Whereas tumor growth was evident in PC (Fig. 2A), PF (Fig. 2B), and DC (Fig. 2C), tumor growth was clearly reduced in the DF group (Fig. 2D). The growth rate was defined as the percentage increase in tumor volume per week (± SE) and was estimated at 16% ± 2% for the PF group (Fig. 2E) in contrast to only 7% ± 1% in the DF group (Fig. 2E). Although statistical significance was not reached between DC group versus DF group (P = 0.13), statistical comparison between PF group (Fig. 2B) versus DF group (Fig. 2D) indicated a highly significant difference in tumor growth rate (P = 0.002). Fenretinide treatment did not affect the growth of the parental xenograft (PC versus PF; P = 0.6; Fig. 2, A and B). However, statistical comparison between the PC (Fig. 2A) and DC group (Fig. 2C) indicated a borderline significance (P = 0.043) suggesting that over a period of 9 weeks there is a possibility that the growth rate of D1 xenograft may be slower than that of parental xenograft, with a growth rate of 11% ± 2% compared with 17% ± 1%, respectively (Fig. 2E). However, inhibition at each dose of fenretinide was calculated relative to the control population. Points are the mean of three separate experiments; bars, ±SD. C, parental and D1 cells were harvested after treatment with increasing dose of fenretinide and cyclin D1 levels determined by Western analysis using anticyclin D1 antibody. D, parental and D1 cells were incubated in the presence or absence of 5 μM of fenretinide for 3, 4, or 5 days. Cells were harvested, fixed in 3.7% paraformaldehyde/PBS, and stained with 4′,6-diamidino-2-phenylindole. The number of apoptotic nuclei in each population was determined. A total of 100 nuclei were counted in three separate experiments; bars, ±SD.
this result needs to be interpreted with care, as a comparison of
the two groups using the nonparametric Mann-Whitney test did
not achieve statistical significance ($P = 0.095$). These observations suggest that fenretinide treatment reduces the rate of tumor
formation arising from xenografts overexpressing cyclin D1, whereas such treatment had no effect on tumor formation arising
from xenografts expressing low levels of cyclin D1.

Fenretinide Treatment Promotes the Regression of Xe-

nograft-overexpressing Cyclin D1. As our results indicated a
preventive effect of fenretinide on the progression of cyclin
D1-overexpressing tumors, we next determined the effect of
fenretinide on tumor regression. For this experiment, cells were
injected and mice divided into four groups as described above.
However, in contrast to the previous experiment, tumors were
allowed to grow without any treatment for a period of 5 weeks.
Statistical analysis of tumor growth between weeks 1 and 5
indicated that cyclin D1-overexpressing xenografts grew faster
then the parental cells (Fig. 3; $P = 0.023$). This result suggested
that the overexpression of cyclin D1 promoted cellular prolif-
eration during the initial establishment of tumor formation com-
pared with an otherwise genetically identical cell population
where cyclin D1 levels are low.
After 5 weeks, fenretinide treatment was initiated. Comparison of tumor growth from weeks 5 to 11 indicated that fenretinide treatment had no significant effect on the tumor growth between the parental groups (Fig. 4, A and B; \( P/H_1^1005 \leq 0.60 \)). However, fenretinide had a drastic effect of the growth of D1 tumors when compared with the mock-treated D1 tumors (\( P/H_1^1021 \leq 0.0005 \); Fig. 4, C and D). In addition, comparison between the PF group (Fig. 4B) and DF group (Fig. 4D) also indicated that fenretinide treatment lead to a drastic reduction in the growth of D1 xenografts compared with parental xenografts (\( P/H_1^1021 \leq 0.0005 \)).

The mean tumor growth rate for the DF group between weeks 5 and 11 (Fig. 4E) showed that fenretinide not only stopped the progression of D1 xenografts but also promoted tumor regression. Therefore, these results indicated that overexpression of cyclin D1 sensitizes cells to fenretinide \( in \text{vivo} \).

Comparison of the tumor growth between DC group (Fig. 4C) and PC group (Fig. 4A) between weeks 5 and 11 also reached statistical significance (\( P = 0.003 \)). Whereas the growth rate of the PC group was \( 18\% \pm 3\% \), that of the DC group was only \( 7\% \pm 1\% \) (Fig. 4F). This result is consistent with our previous observation of an apparent slower rate of tumor growth in the D1 group compared with parental group (Fig. 2E). Therefore, we conclude that although the initial growth rate of D1-overexpressing tumors was faster before week 5, after a longer period of time (week 11) overexpression of cyclin D1 has a negative effect on cellular proliferation.

Expression of Free Cyclin D1 Enhances the Sensitivity to Fenretinide. The role of cyclin D1 in complex with cdk4 is well known to promote cellular proliferation by leading to entry into S phase. However, several reports have described distinct roles for cyclin D1 independently of its binding to cdk4 that may contribute to its oncogenic action (19, 20). To determine whether the interaction between fenretinide treatment and cyclin D1 is dependent on its binding to cdk4, we created T47-D-based stable clones overexpressing a mutant form of cyclin D1 (cyclin D1-KE) that cannot bind to cdk4. The resulting cell line was named D1-KE and used in a tumor regression experiment, in which either D1 cells or D1-KE cells were injected into nude mice and tumors allowed to grow for 5 weeks before treatment. No significant difference in the growth rate was observed between DC xenografts (Fig. 5A) and D1-KEC (Fig. 5C; \( P/H_1^1005 \leq 0.92 \)). However, a highly
significant reduction in growth rate was observed between D1-KEC (Fig. 5C) and D1-KEF (Fig. 5D) with a P of <0.0005 and a mean growth rate of 15% compared to −9%, respectively (Fig. 5E). Furthermore, statistical analysis between the DF group and the D1-KEF group suggested that tumor regression in the D1-KEF group was more pronounced then in the DF group (P = 0.033) as indicated by a mean growth rate of −9% and −1%, respectively. These results indicate that the cooperation between cyclin D1 and fenretinide treatment is independent of cyclin D1 binding to cdk4, and suggests that free cyclin D1 is responsible to generate a signal that lowers the threshold of fenretinide required to induce apoptosis.

**Fenretinide Promotes Tumor Regression of MCF-7 Xenograft.** MCF-7 cells overexpress cyclin D1 because of amplification of the cyclin D1 gene (21). As our data suggest that T47-D cells that express low levels of cyclin D1 are not sensitive to fenretinide treatment but that forcing the overexpression of cyclin D1 in these cells sensitize them to fenretinide treatment, we next tested the effect of fenretinide on MCF-7 cells. Nude mice were injected with MCF-7 cells, and tumors were allowed to form for 3 weeks. As expected, no difference in the tumor growth rate was observed between mice assigned to the MCF-7 control group and the MCF-7-treated group between weeks 1 and 3. At week 3, treatment was initiated and tumor volume measured weekly. As observed for the D1 cells, fenretinide treatment led to the regression of MCF-7 xenografts (Fig. 6B) compared with the control group (Fig. 6A) as reflected by the mean growth rate of −2% and 14%, respectively. This result indicates that fenretinide promotes the regression of MCF-7 xenografts, a cell line that naturally overexpresses cyclin D1.

**DISCUSSION**

Cyclin D1 is overexpressed in 35% of breast cancers. Cyclin D1 was found recently to be essential for tumor formation in MMTV-erb-2 and MMTV-ras transgenic mice (10). This important finding indicated a significant role for cyclin D1 in breast cancer and immediately raised the possibility that drugs able to synergize with cyclin D1 may be very useful for the treatment of this significant subset of breast cancers. Fenretinide shows promising results for the chemoprevention of breast cancer (17). However, in this trial, patients were not selected for a specific molecular marker to predict response; therefore, it is possible that only a few patients would have expressed cyclin D1 in their tumors. Our results shown here indicate that overexpression of cyclin D1 sensitizes breast cancer cells to killing...
by fenretinide. This finding is consistent with the observation that fenretinide delays the formation and reduces the size of tumors arising in MMTV-erb-2 transgenic mice, as these tumors overexpress cyclin D1 (9).

The mechanism of synergy between cyclin D1 overexpression and fenretinide remains unclear; however, the observation that the levels of exogenously expressed HA-cyclin D1 remain elevated in D1 cells after treatment with fenretinide (Fig. 1C) rules out the possibility that viability in the overexpressing cells is dependent on cyclin D1. Overexpression of cyclin D1 in cells in the absence of serum induces apoptosis, and this is thought to be the result of conflicting cell signaling (22). Although the precise mechanism underlying this effect remains undefined, the observation that overexpression of cyclin D1 sensitizes breast cancer cells to ATRA-induced apoptosis, a ligand that is normally a very poor inducer of apoptosis, is consistent with the ability of cyclin D1 to induce apoptosis under specific conditions. Because fenretinide, but not ATRA, can be used for the treatment of breast cancer, our data offers the first clinically relevant condition in which the effect of cyclin D1 on apoptosis is observed.

Furthermore, our results show that cyclin D1 binding to cdk4 is not required for the interaction between fenretinide and cyclin D1 to be observed. Therefore, free cyclin D1 appears to generate the signaling event that lowers the threshold of fenretinide-induced apoptosis and argues in favor of a model whereby the cooperation observed between cyclin D1 and fenretinide is linked to a negative role of cyclin D1 on cellular proliferation that is unrelated to its binding to cdk4. In agreement with such a model is our finding that cyclin D1 overexpression alone reduces tumor growth rate over an extended period of time in vivo (compare Fig. 4A with Fig. 4C). Furthermore, this observation may also offer a potential explanation to the surprising report of a better survival rate for breast cancer patients with elevated levels of cyclin D1 in their tumors (23).

We found that fenretinide treatment of xenografts overexpressing cyclin D1 is able to prevent tumor formation. These data are consistent with the indication of a benefit of fenretinide treatment in the prevention of breast cancer recurrence in premenopausal women (17). Whether the tumors of patients that did not recur after fenretinide treatment overexpressed cyclin D1 would have been very interesting to determine. However, the estimation of the statistical power of such study was low, and, therefore, could not reach statistical significance. Therefore, a new trial would be necessary to determine whether cyclin D1 overexpression predicts a positive response to fenretinide treatment. The importance of selecting patients for specific molecular marker is best illustrated by the fact that in a trial including 100 patients with a target response rate of 60% but in which only 10% of tumors express the appropriate molecular marker, the observed response rate will be only 6%. However, if patients are selected such that 100% of the patients express the molecular marker, the success rate of the trial is increased to 60%.

We also showed that fenretinide is able to promote the regression of xenografts overexpressing cyclin D1. Considering that fenretinide has been studied mostly in tumor prevention trials, this is potentially important as it suggests that fenretinide may be useful not only in preventing tumor recurrence, but also in the treatment of palpable tumors, at least potentially in the subpopulation of tumors that show elevated levels of cyclin D1. In conclusion, we have reported for the first time a positive interaction between fenretinide treatment and overexpression of cyclin D1. Fenretinide has the immense advantage of resulting in very few adverse side effects, to have a stable plasma con-
centration and to concentrate in the breast. Furthermore, overexpression of cyclin D1 has been proposed to promote resistance to Tamoxifen (24), an observation that emphasizes the importance of identifying therapeutic alternatives for the treatment of cyclin D1-overexpressing tumors. Therefore, considering the clinical advantages that fenretinide offers and the high percentage of breast cancers that show abnormal elevation in cyclin D1 levels, the interaction between cyclin D1 and fenretinide represents a promising step toward the design of a nontoxic and targeted therapy for breast cancer treatment.

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