Altered localization of a coactivator sensitizes breast cancer cells to tumor necrosis factor–induced apoptosis

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Abstract

Proline-, glutamic acid-, and leucine-rich protein-1 (PELP1) is a novel coregulator of the estrogen receptor that plays a role in both genomic and nongenomic actions of the estrogen receptor. Emerging studies suggest that in addition to the nuclear localization of PELP1, it is predominantly localized in the cytoplasm in human breast tumors, leading to excessive nongenomic signaling and possibly to tamoxifen resistance. The mechanisms underlying resistance to hormones in preclinical model systems remain under intense investigation. In an effort to develop a model system to treat tumor cells with cytoplasmic PELP1 expression and tamoxifen resistance, here we used the cytokine tumor necrosis factor (TNF)-α. We found that clones of MCF-7 human breast cancer cells overexpressing PELP1 in the cytoplasm were distinctly sensitive to TNF-α-induced apoptosis than were wild-type nuclear PELP1+ and pcDNA vector–expressing clones as revealed by cell growth assay, cell cycle analysis, Annexin V staining, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. We also found that the clones with cytoplasmic PELP1 overexpression had significantly less antiapoptotic protein Bcl-2 and nuclear factor-κB DNA binding, but increased cyclin E expression, further supporting evidence that these cells are sensitive to apoptosis. The mechanism behind TNF-induced apoptosis in these cells involves caspasas, as revealed by poly(ADP-ribose) polymerase cleavage and the broad-spectrum caspase inhibitor Z-VAD-inhibited apoptosis. In conclusion, our results suggest that altered localization of PELP1 promotes heightened sensitivity to TNF-α in MCF-7 cells, paving the way for developing new treatment strategies for tumors with cytoplasmic PELP1 expression. [Mol Cancer Ther 2006;5(2):230–7]

Introduction

Endocrine therapies for breast cancer have been designed to interrupt estrogen signaling by either blocking the estrogen receptor (ER) or reducing the amount of estrogen available for binding in the cell. The antiestrogen tamoxifen is effective against ER-positive breast cancers, slowing tumor growth and preventing disease recurrence. However, many patients who initially respond to tamoxifen later develop resistance to antiestrogens and selective ER modulators (1). Several mechanisms of resistance to hormonal therapy have been proposed, including expression of variant or mutant ER, ligand-independent activation of ER, adaptation of tumors to lower concentrations of estrogen, and other pharmacologic changes (2, 3). Existing and emerging data also suggest that ER coregulators play a role in hormone responsiveness and tumor progression (4, 5). However, the causes of ER coregulator–linked resistance to hormonal therapy and possible interference with this phenomenon remain elusive.

ER, a ligand-dependent transcription factor, modulates transcription of a number of genes and contributes to genomic responses (6). Recent studies suggested that in addition to its well-studied nuclear functions, ER participates in cytoplasmic signaling pathways (nongenomic signaling; refs. 7, 8). Nongenomic signaling has been linked with the rapid responses to estrogen and generally involves stimulation of the Src, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, and protein kinase C pathways in cytosol (9, 10). AIB1, also known as steroid receptor coactivator 3, is a member of the p160 family of nuclear receptor coactivators. It is localized in both the cytoplasm and nuclear compartments of breast cancer cells. In particular, AIB1 nuclear localization is associated with the ER-α status, and patients with breast cancer with AIB1 nuclear expression tend to have a good response to hormonal therapy when compared with those with intense cytoplasmic expression (11). More recently, work from this laboratory has shown that proline-, glutamic acid-, and leucine-rich protein-1 (PELP1), a molecule with both genomic and nongenomic functions in breast cancer cells, plays an important role in antiestrogen resistance. PELP1 is a novel steroid receptor coregulator with 10 nuclear receptor LXXLL motifs, participates in both transcriptional regulation and nongenomic stimulation of Akt and MAPK, and is localized in both the nuclear and cytoplasmic compartments of breast cancer cells. The mechanisms underlying resistance to hormones in preclinical model systems remain under intense investigation.
compartment (12–14). It is identical to the modulator of nongenomic activity of ER, which has been implicated in nongenomic stimulation of the Src and MAPK pathways by ER. PELP1 is localized predominantly in the cytoplasm in a significant proportion of human breast (15), endometrial (14), and salivary gland (16) carcinomas. Therefore, PELP1-induced nongenomic signaling is expected to have physiologic implications in breast cancer tumorigenesis. However, the significance of cytoplasmic coactivators in nongenomic signaling is beginning to surface and their role in resistance to selective ER modulators remains unknown.

There are two approaches to dealing with hormone resistance caused by cytoplasmic PELP1: restoration of hormone sensitivity, which by itself has its own limitations, and confirmation that cells with cytoplasmic PELP1 can be sensitive to cytotoxic therapeutic agents, such as tumor necrosis factor (TNF)-α. TNF-α is recognized as one of the most pleotropic cytokines as it acts as a cytotoxic agent against a variety of tumor cell lines (17). TNF-α is capable of inducing tumor regression with efficiency comparable with that of chemotherapeutic agents and plays a major role in tumor regression (18). In the present study, we used the model human breast cancer cell line MCF-7, which specifically expresses PELP1 in the cytoplasm (15). We found that these cells are sensitive to TNF-α-induced apoptosis than are cells with nuclear PELP1. Our results indicate that TNF-α could be used as an alternate treatment agent to tamoxifen in patients with breast cancer with cytoplasmic expression of PELP1.

Materials and Methods

Cell Cultures and Reagents

MCF-7 human breast cancer cells overexpressing wild-type PELP1 (12), cytoplasmic PELP1 (15), and a pcDNA vector were maintained in DMEM-F12 (1:1) supplemented with 10% FCS. Charcoal-stripped serum (DCC serum), TNF-α, tamoxifen, Z-VAD, and antibodies against β-actin were purchased from Sigma Chemical Co. (St. Louis, MO). An anti-Bcl-2 antibody was obtained from DAKO (Carpinteria, CA), and anti-poly(ADP-ribose) polymerase (PARP) antibody was purchased from BD Biosciences PharMingen (San Jose, CA). An anti-T7-epitope antibody was purchased from Novagen (Milwaukee, WI). Antibodies against phospho-AKT and phospho-MAPK were purchased from Cell Signaling Technology (Beverly, MA). Antibodies for cyclin E were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Reverse transcription-PCR for cyclin E and glyceraldehyde-3-phosphate dehydrogenase was done using the Access reverse transcription-PCR system (Promega, Madison, WI) according to the instructions of the manufacturer.

Cell Extracts, Immunoblotting, and Immunoprecipitation

Cell lysates were prepared with Triton X-100 buffer [50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 0.5% Triton X-100, 1× protease inhibitor mixture, 1 mmol/L sodium vanadate] for 15 minutes on ice. The lysates were centrifuged in an Eppendorf centrifuge at 4°C for 15 minutes. Cell lysates containing equal amounts of protein (~200 μg) were resolved on SDS-polyacrylamide gels (8% acrylamide), transferred to nitrocellulose membranes, probed with the appropriate antibodies, and developed by using either enhanced chemiluminescence or alkaline phosphatase color-based reaction.

Cell Proliferation Assay

For cell proliferation assays involving TNF treatment, equal numbers of cells were plated in triplicates in DMEM supplemented with 10% serum and then treated with TNF-α (10 ng/mL) and Z-VAD (50 μmol/L) as indicated. The proliferation rate of the cells was measured after 24 or 48 hours by counting with a Coulter Counter (Beckman Coulter, Fullerton, CA).

Cell Cycle Analysis

For cell cycle experiments, equal numbers of cells were plated in triplicates, treated with TNF-α (10 ng/mL) for 12 hours, and fixed in 70% ethanol. Cells were treated with 500 μg/mL RNase, stained with 10 μg/mL propidium iodide (Sigma-Aldrich, St. Louis, MO) and analyzed by using a flow cytometer.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was done with cells plated on coverslips according to the instructions of the manufacturer (in situ Cell Death Detection kit, Roche Diagnostics, Penzberg, Germany). Both light and fluorescent images were obtained. More than five representative fields were analyzed per slide and 25 cells per field were analyzed from the stored images. Statistical analysis was done from three independent experiments.

Annexin V FITC Assay

The Annexin V assay was conducted with the use of a FITC-labeled Annexin V apoptosis kit (Sigma Chemical). In brief, cells were plated in 24-well plates at a density of 1 × 10⁴ per well. On the next day, the medium was replaced with fresh medium and cells were exposed to 10 ng/mL TNF-α for 12 hours. At the end of the experiment, FITC-labeled Annexin V was added to the culture medium to a final concentration of 1 μL/mL in the presence of 0.2 mmol/L CaCl₂, and the cells were incubated for 10 minutes at 37°C. Apoptotic cells were identified by direct visualization of green plasma membrane staining under a fluorescence microscope. Both light and fluorescent images were obtained. Generally, more than five representative fields in each well were analyzed and 25 cells per field were analyzed from the stored images and statistical analysis was done.

Electrophoretic Mobility Shift Assay

To determine the level of nuclear factor κB (NF-κB) binding, we did electrophoretic mobility shift assay as described previously (19) by using oligonucleotides for NF-κB purchased from Santa Cruz Biotechnology. Briefly, the NF-κB double-stranded oligonucleotide corresponding to the NF-κB consensus sequence (5'-AGTTAGGGCGACTTTCCCAGGC-3') was end labeled with [γ-32P]ATP and T4 DNA Ligase. The labeled oligonucleotide was incubated with nuclear extracts from MCF-7 cells for 15 minutes on ice. The complexes were resolved on a 4% native polyacrylamide gel.
polynucleotide kinase and purified with G-25 columns. Nuclear extract prepared was incubated with labeled NF-κB oligonucleotide in binding buffer for 30 minutes at 37°C, and the DNA-protein complex were resolved on a 7.5% native polyacrylamide gel. The radioactive bands from the dried gels were visualized and quantitated by using a Phosphorimager (Molecular Dynamics/Amersham Biosciences, Sunnyvale, CA) using ImageQuant software.

**Statistical Analysis**

Data were analyzed using PRISM software (GraphPad Software, Inc., San Diego, CA). Pairwise analysis of the effect of TNF on cell number in individual cell lines in Figs. 1A and 5A were assessed using Student’s t test. All other analyses used a parametric ANOVA test for overall significant differences followed by Tukey’s multiple comparison test for pairwise analyses of differences within an experiment. Significance was accepted if $P < 0.05$.

**Results**

Recent studies from our group (15) showed that cytoplasmic localization of PELP1 promotes resistance to tamoxifen. Therefore, we sought to devise a strategy for treating the MCF-7 cells with cytoplasmic PELP1 expression and screened with resveratrol and doxorubicin to test their efficiency to inhibit the growth of such cells. Cytoplasmic PELP1–expressing clones showed resistance to these drugs (data not shown). Because TNF family of cytokine agents is capable of directly inducing tumor regression with an efficiency comparable with that of chemotherapeutic agents, we used TNF-α as proof of principle to target these cells.

Interestingly, results of a cell proliferation assay showed that cytoplasmic PELP1–expressing clones were sensitive to TNF-α-induced apoptosis and showed an almost 50% reduction in cell number when compared with wild-type PELP1 and pcDNA-expressing clones (Fig. 1A and B).

To validate the above results, we assessed TNF-α-induced apoptosis by Annexin V staining, which detects early events...
in apoptosis. Figure 2 shows that after 12 hours of TNF-α treatment, cytoplasmic PELP1–expressing clones displayed strongly increased Annexin V staining. We observed no apoptotic cells in either wild-type PELP1 or pcDNA-expressing clones. Because TNF-α can induce cell death by both apoptosis and necrosis, to further show that TNF-induced cell death is caused by apoptosis, we carried out the TUNEL assay. As indicated by the number of fluorescence-positive cells in cytoplasmic PELP1–expressing clones, there was an increase in apoptosis in these clones compared with wild-type PELP1 and pcDNA-expressing clones (Fig. 3). Further, cell cycle analysis of cytoplasmic PELP1–expressing clones treated with TNF showed that nearly 10% of the total cells analyzed were in the pre-G0 phase of the cell cycle, providing proof of principle that cytoplasmic localization of PELP1 sensitizes cells to TNF-induced apoptosis (Fig. 4).

To determine whether caspases are involved in TNF-α–induced apoptosis of cytoplasmic PELP1–expressing clones, cells were preincubated for 1 hour with Z-VAD, a broad-spectrum caspase inhibitor, and then treated for 48 hours with TNF-α. As shown in Fig. 5A and B, Z-VAD abrogated the apoptotic effect of TNF-α in cytoplasmic PELP1–expressing clones in cell proliferation assay. This showed that TNF-induced apoptosis requires activation of members of the caspase family of proteases. Activation of a caspase cascade leads to cleavage of cellular proteins, such as the DNA repair enzyme PARP; this cleavage results in inactivation of the enzyme. TNF induced PARP cleavage only in cytoplasmic PELP1–expressing clones, and we observed the 85 kDa cleavage product as early as 12 hours after treatment (Fig. 5C).

The ratio of proapoptotic proteins to antiapoptotic proteins is thought to be an important factor in the execution of the programmed cell death pathway. Recent studies suggested that cytoplasmic PELP1 promotes activation of the MAPK and Akt pathways (15), which are

Figure 3. A, TUNEL assay on cytoplasmic PELP1–expressing clones showing fluorescent apoptotic cells 24 h after TNF treatment. Both positive and negative controls were included in the test. One-way ANOVA analysis was done with Tukey’s multiple comparison test. Columns, mean; bars, SE (n = 3). TNF caused an increase in the levels of apoptosis in PELP1-cyto clones (P < 0.01). B, companion fluorescent image (middle), bright field image (left), and overlay (right) of a single representative data.
implicated in cell survival. Because a number of studies were based on the possibility of sensitizing cells to undergo apoptosis by reducing the levels of antiapoptotic proteins, we next checked the level of Bcl-2 protein expression in these cells before and after treatment with TNF-α. Western blot analysis indicated that cytoplasmic PELP1−expressing clones had very less Bcl-2 protein expression than did wild-type PELP1− or pcDNA-expressing clones (Fig. 5C). Therefore, we hypothesized that a decrease in Bcl-2 expression and an increase in Akt and MAPK activation alters the ratio of proapoptotic proteins to antiapoptotic proteins, making cells more susceptible to TNF. This suggested that deregulation of proapoptotic and antiapoptotic proteins is a component of heightened sensitivity to cytokines.

Because NF-κB plays a key role in the suppression of TNF-mediated apoptosis (20), we next analyzed the binding of PELP1 to NF-κB consensus sequence oligonucleotide by using electrophoretic mobility shift assay. Results showed that in cytoplasmic PELP1−expressing clones, the binding of NF-κB to its consensus DNA binding site was reduced than in wild-type PELP1− and pcDNA-expressing clones (Fig. 6A). This further supported the notion that these cells are sensitive to TNF-mediated apoptosis.

Because earlier studies have shown a correlation between TNF-α-mediated apoptosis and increased cyclin E overexpression in MCF-7 cells (21), we next examined the levels of cyclin E in the clones used above. Results showed that cytoplasmic PELP1−expressing clones have higher levels of cyclin E protein, but not cyclin E mRNA, compared with wild-type PELP1 and pcDNA-expressing clones (Fig. 6B). This indicates that cytoplasmic localization of PELP1 leads to up-regulation of cyclin E protein, which in turn could sensitize cells to TNF-induced apoptosis.

**Discussion**

Cell sensitivity to TNF-α and altered localization of coactivators were important issues under intense investigation in this study. Results reported here showed that cytoplasmic localization of ER coactivator PELP1 sensitizes breast cancer cells to TNF-α-induced apoptosis, providing clues to alternate therapeutic strategy.

ER coregulatory proteins have been suggested to play a role in observed tissue-specific effects of tamoxifen (22, 23). ER coregulators are targeted by excessive ER-HER2 cross-talk leading to hormonal resistance in a subset of breast tumors (24). High levels of expression of the ER coactivator AIB1 and HER2 in breast cancer contribute to tamoxifen resistance (25). Also, ER function is modulated by interactions with coactivators and corepressors. Tamoxifen inhibits ER transcriptional activity by competitively inhibiting estradiol binding and inducing conformational changes in the receptor that may prevent its interaction with coactivators. Although tamoxifen had been the unchallenged standard of treatment in women with hormone-receptor−positive invasive breast carcinoma until recently, the mechanisms of its action and acquired resistance to it during treatment are largely unknown. The novel ER coregulator PELP1 (also known as modulator of nongenomic activity of ER) plays a role in both genomic and nongenomic actions of ER. PELP1 is predominantly localized in the nucleus in hormonally responsive tissues, but recent studies suggest that PELP1 may be localized exclusively in the cytoplasm in cancer cells (14–16). Earlier studies by our group suggested that ER-positive breast cancer cells with overexpression of PELP1 in the cytoplasm (cytoplasmic PELP1−expressing clones) are resistant to tamoxifen. The mechanisms of this resistance are not yet clear. However, as an alternate strategy to treating such cells with cytoplasmic PELP1, we explored the possibility of using TNF-α because of its potential as an anticancer agent.

In previous studies, MCF-7 cells reportedly responded only weakly to TNF-α (26, 27). The reported differences in the sensitivity of MCF-7 cells to TNF-α raised the possibility that variations in MCF-7 cell strains among laboratories may account for these discrepancies. TNF-α is an effective inhibitor of proliferation and inducer of apoptosis of MCF-7 cells (28, 29). We used a single MCF-7 strain available in our laboratory to generate all three stable clones (cytoplasmic-PELP1, wild-type PELP1− and pcDNA-expressing clones) and observed that cytoplasmic PELP1−expressing clones are sensitive to TNF-α-induced apoptosis than the other two clones in cell proliferation...
assays, Annexin V staining, cell cycle analysis, and TUNEL assay. In addition, expression of Bcl-2 family members determines the survival or apoptosis of cells mainly by preventing the release of cytochrome c from mitochondria (30). The reduction in the expression of Bcl-2 protein in the cytoplasmic PELP1–expressing clones further explains the heightened sensitivity to TNF-induced apoptosis.

Because NF-κB regulates many survival genes (31, 32), it is not surprising that NF-κB activation has been implicated in breast cancer chemotherapy resistance mechanism. Previous reports suggest that activation of NF-κB via the phosphatidylinositol 3-kinase/Akt signaling pathway may be a significant mechanism for development of endocrine therapy resistance in breast cancer and that inhibition of NF-κB activity either pharmacologically or by small molecular approach restored hormone sensitivity (33–39). Inhibition of NF-κB seems to be a promising approach to increase the cytotoxic effects of anticancer agents. Despite all this, activation of NF-κB has been linked to apoptosis, either as an antiapoptotic or proapoptotic (40), depending on the cell type in which it is expressed and furthermore, it is possible that cyto-PELP1 may further influence the biological outcome of NF-κB. Reduced binding of NF-κB to its consensus DNA binding site in these cells supports the hypothesis that they are more susceptible to apoptosis. Finally, activation of caspases, a family of cysteine proteases, is important for cell death induced by TNF (41, 42). Because MCF-7 cells do not express caspase-3 protein, other caspases may substitute for caspase-3. Indeed, our results showed that apoptosis was inhibited by Z-VAD, implying that this effect is caspase-dependent. However, our present study was limited to the use of MCF-7 cells.

Because cytoplasmic localization of PELP1 leads to up-regulation of cyclin E, it is possible that the hypersensitivity of the cytoplasmic PELP1–expressing cells may result from the noted up-regulation of cyclin E protein as shown before (21) or an increased proliferation or both. However, because we did not observe any change in the levels of cyclin E mRNA, it seems that the observed increased cyclin E protein may not be a consequence of the putative effects of PELP1 on the cell cycle and that cytoplasmic PELP1 may directly or indirectly influence the levels of cyclin E via a posttranscription mechanism, which is yet-to-be identified. Overall, these findings provide reasonable evidence in support of sensitivity effect of cytoplasmic PELP1–expressing cells to cytotoxic agent, such as TNF-α.

Despite the systemic side effects of TNF family, several cytokines have been tested as antineoplastic agents both alone and in combination with conventional chemotherapeutic agents (43–45). However, the role of TNF-α in the

Figure 5. TNF-α-induced apoptosis is caspase dependent. A and B, cytoplasmic PELP1 – expressing clones were preincubated with Z-VAD (50 μmol/L) for 1 h before the addition of TNF-α for 2 d, and the cell number was determined. Columns, mean (n = 3); bars, SE. Cell number was reduced in PELP1-cyto clones compared with the control cells treated with TNF and showed no decrease in cell number when treated with Z-VAD (P < 0.01, Student’s t test). Photographs were taken under a phase contrast microscope and are representative of the results of three separate experiments. C, TNF-α-induced PARP cleavage in cytoplasmic PELP1 –expressing clones. pcDNA-, wild-type PELP1 –, and cytoplasmic PELP1 –expressing clones were treated with or without TNF-α (10 ng/mL) for 12 and 24 h, protein lysates were subjected to Western blot analysis and probed with antibodies against PARP, Bcl-2, phospho-Akt, and phospho-MAPK. Bottom, quantitation of the 85 kDa cleaved PARP fragment from three independent experiments.
treatment and pathogenesis of cancer remains poorly understood. Recently, many approaches have been used to target therapeutic TNF-α directly to tumors without systemic toxicity (46–49). TNF-α mediates tumor regression and recombinant TNF-α has been approved in Europe for local regional administration at supraphysiological levels as therapy for sarcoma (50). In summary, our findings show the existence of a close relationship between cytoplasmic PELP1 localization and increased TNF-α sensitivity and bring to light that TNF-α, in combination with chemotherapeutic agents, can be potentially considered as an alternate therapeutic strategy in the near future for tumors with cytoplasmic PELP1 expression that are resistant to several chemotherapeutic drugs. However, the therapeutic value of TNF-α for cancer seems to be limited by both its toxic effects on normal tissues and the wide variation in TNF sensitivity of different tumor cells (51). We believe that these findings presented here offer the proof-of-principle in support of achieving antitumor effects in tamoxifen-resistance breast cancer cells with cytoplasmic PELP1, a physiologic situation in the human breast tumors, by the TNF family of cytokines.

References

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