Reduced PTEN expression in breast cancer cells confers susceptibility to inhibitors of the PI3 kinase/Akt pathway

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The PTEN protein is a lipid phosphatase with putative tumor suppressing abilities, including inhibition of the PI3K/Akt signaling pathway. Inactivating mutations or deletions of the PTEN gene, which result in hyper-activation of the PI3K/Akt signaling pathway, are increasingly being reported in human malignancies, including breast cancer, and have been related to features of poor prognosis and resistance to chemotherapy and hormone therapy. Prior studies in different tumor models have shown that, under conditions of PTEN deficiency, the PI3K/Akt signaling pathway becomes a fundamental proliferative and survival pathway, and that pharmacological inhibition of this pathway results in tumor growth inhibition. This study aimed to explore further this hypothesis in breast cancer cells. To this end, we have determined the growth response to inhibition of the PI3K/Akt signaling pathway in a series of breast cancer cell lines with different PTEN levels. The PTEN-negative cell line displayed greater sensitivity to the growth inhibitory effects of the PI3K inhibitor, LY294002 and rapamycin, an inhibitor of the PI3K/Akt downstream mediator mTOR, compared with the PTEN-positive cell lines. To determine whether or not these differences in response are specifically due to effects of PTEN, we developed a series of cell lines with reduced PTEN protein expression compared with the parental cell line. These reduced PTEN cells demonstrated an increased sensitivity to the anti-proliferative effects induced by LY294002 and rapamycin compared with the parental cells, which corresponded to alterations in cell cycle response. These findings indicate that inhibitors of mTOR, some of which are already in clinical development (CCI-779, an ester of rapamycin), have the potential to be effective in the treatment of breast cancer patients with PTEN-negative tumors and should be evaluated in this setting.

Key words: breast cancer, PI3K/Akt, PTEN

Introduction

The tumor suppressor PTEN/MMAC1/TEP1 (PTEN, phosphatase and tensin homolog deleted from chromosome 10; MMAC 1, mutated in multiple advanced cancers 1; TEP1, TGFB-regulated and epithelial cell-enriched phosphatase 1) is a lipid phosphatase which is a physiological inhibitor of the phosphatidylinositol (3,4,5)-triphosphate kinase (PI3K)/protein kinase B (Akt) signal transduction pathway. The PTEN gene is frequently mutated or inactivated in a high proportion of human cancers, including up to 30% of breast cancers, resulting in hyper-activation of the PI3K/Akt signaling pathway. In addition, germline mutations in the PTEN gene are associated with multi-neoplastic, autosomally dominant syndromes in humans, such as Cowden’s disease and Bannayan–Zonana syndrome, which feature a predisposition to formation of several different malignancies, including breast cancer (20–50% of affected females) [1, 2]. Cell lines with defective PTEN have alterations in the cell cycle regulation and a defective apoptotic response [reviewed in 3]. Studies in breast cancer indicated a correlation between defective PTEN and features of poor prognosis as well as resistance to hormone therapy [4, 5]. Furthermore, adenoviral transfer of the PTEN gene to PTEN-defective cells results in restoration of chemosensitivity and has been demonstrated to have antiproliferative effects [6, 7].

Although the intracellular functions of PTEN are complex and only partially deciphered, it appears that one of the most relevant consequences of the PTEN-defective phenotype is the activation of the PI3K/Akt signaling pathway. Akt mediates multiple intracellular functions pertaining to cell proliferation and apoptosis and has been implicated in chemoresistance in colon, bladder and ovarian cancers [7–9]. In addition, recent
studies suggest that pharmacological or genetic modulation of Akt activity alters the response of non-small cell lung cancer (NSCLC) cell lines to conventional chemotherapy [10]. Although the signaling pathways downstream from Akt are not totally elucidated, it is well established that mTOR (also known as FRAP and RAFT) is one of the most relevant mediators of Akt functions [11]. mTOR is activated in response to Akt phosphorylation, resulting in the phosphorylation of p70S6 kinase and 4E-BP1, and, consequently, increases the translation of mRNAs of proteins involved in the regulation of the cell cycle [12]. Recently, PTEN-negative cell lines have been shown to have elevated p70S6 kinase activation that is abrogated in the presence of the mTOR inhibitor CCI-779, an ester of rapamycin, supporting the notion that mTOR is an important downstream signaling mediator in PTEN-negative tumors [13, 14]. Inhibition of mTOR by rapamycin results in cell cycle arrest, p53-dependent and -independent apoptosis and tumor growth inhibition [15–17]. CCI-779, an ester of rapamycin, is currently in clinical development for the treatment of cancer. Preliminary data from ongoing clinical trials indicate that CCI-779 is well tolerated and has significant antitumor activity in a variety of tumor types, including breast cancer [18].

We have been interested in developing specific treatments for patients with PTEN negative tumors. In this study, we assessed the response of a series of breast cancer cell lines with varied PTEN levels to the mTOR inhibitor rapamycin and other inhibitors of the PI3K/Akt pathway. We found that PTEN status negatively correlated with sensitivity to the growth inhibitory effects of rapamycin and the PI3 kinase inhibitor LY294002. These findings support previous observations and suggest that inhibitors of mTOR could prove especially effective in the treatment of tumors with a PTEN-negative phenotype.

Materials and methods

Cell lines

MCF-7, MDA-MB-435 and MDA-MB-68 breast cancer cells were obtained from the American Type Culture Collection and maintained in Improved Minimal Essential Medium (IMEM) (Gibco/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Sigma, St Louis, MO) and 6 ng/ml of bovine insulin (Sigma).

Western blot analysis

Cells were seeded in 6-well plates at a density of \(2 \times 10^5\) cells per well. Cells were serum-starved for 18 h, then stimulated with 10% FBS for the indicated times. Cells were then harvested in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors and 0.1 M sodium orthovannadate in PBS) and 40 μg samples of protein extract were resolved on a polyacrylamide gel. These were subsequently transferred from the gel to nitrocellulose membranes and subjected to immunodetection with antibodies against PTEN (Sigma), phosphorylated and total Akt, phosphorylated and total p70S6 kinase (Cell Signaling Technology, Beverly, MA) and finally actin (Santa Cruz Biotechnology, Santa Cruz, CA) for a loading control. Signal was detected using the enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL).

Growth proliferation assay

Cell growth was assessed by MTT [3,4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide] (Sigma) dye conversion at 570 nm following the manufacturer’s instructions. Briefly, cells were seeded 5 × 10^4 per well in a 96-well flat bottom plate. Cells were allowed to grow for 24 h, then placed in serum-starved conditions for 18 h. Cells were then treated for 96 h with indicated concentrations of LY294002, rapamycin and PD98059 (all from Sigma) in the presence of 10% FBS. 20 μl of MTT (5 mg/ml in PBS) was then added to each well. After 3 h incubation at 37°C, cells were lysed by the addition of 0.1 N HCl in isopropanol.

Expression vectors and constructs

The expression plasmid for PTEN, CMV-PTEN, has already been described [19]. For the antisense (AS) PTEN plasmid, forward (5’-GGTT-CGAACGGCCGCTGAGCTCCA-3’) and reverse (5’-CCAGATTTTTC-TCAGTTTATTCAAGTTTATTTTC-3’) primers were designed with HindIII and XhoI restriction digest sites, respectively, for ease of cloning, and used to amplify a 1.7 kb fragment of the human PTEN cDNA from the CMV-PTEN plasmid. This cDNA fragment was cloned into the pCDNA3.1(−) expression vector (Invitrogen, Carlsbad, CA) in the antisense orientation. All constructs were confirmed by dideoxy sequencing.

Generation, selection and analysis of stable transfectants

MCF-7 cell lines were transfected with the antisense PTEN constructs or with an empty plasmid as a control, using FuGene 6 (Boehringer Mannheim, Indianapolis, IN). One day after transfection, cells were placed into the selection medium containing 1.0 mg/ml G418 (Gibco/BRL). Twenty-one days after selection, individual G418-resistant colonies were subcloned. PTEN protein expression was analyzed by western blot using the above described PTEN antibody. Several clones were selected based upon PTEN expression levels. Figures are shown with data from two representative clones of several selected.

Flow cytometry

Flow cytometry analysis was performed as previously described [20], with the following modifications: the propidium iodide was in the presence of PBS with 25 mg/ml RNase A and 0.5% Triton X-100.

Data analysis

Each growth inhibition or flow cytometry experiment was repeated 5–6 times and the average data is presented. For growth inhibition studies, values were expressed as a percentage of untreated controls from which IC_{50} concentrations were calculated and mean values compared using non-parametric tests for two or multiple unrelated samples. The same methodology was used to compare the flow cytometry analysis data.

Results

Correlation of PTEN status with response to PI3K/Akt pathway inhibitors

In order to determine the relationship between PTEN status and response to inhibitors of the PI3K/Akt kinase pathway, we first screened several breast cancer cell lines by western blot analysis for expression of PTEN, as well as the phosphorylation status of two of its downstream targets. In this paper, we have presented results from cell lines representing various levels of...
PTEN status. These include the estrogen receptor-negative MDA-MB-435 and MDA-MB-468 cell lines, and the estrogen receptor-positive MCF-7 cell line. These cell lines have been used extensively in preclinical studies of breast cancer and have been shown to translate well to clinical results. As seen in Figure 1A, both the MDA-MB-435 and MCF-7 cell lines expressed PTEN, while the MDA-MB-468 cells had no detectable levels. The MDA-MB-435 cells had no detectable phosphorylated Akt, even after stimulation with 10% FBS for up to 60 min. The MCF-7 cells had little activated Akt in extracts from unstimulated cells, but did have measurable levels of phosphorylated Akt after treatment with 10% FBS. Not surprisingly, the PTEN-negative MDA-MB-468 cells displayed constitutively phosphorylated Akt. Total Akt protein remained unchanged in each of the cell lines tested. Levels of phosphorylated p70S6 kinase, a downstream target of Akt, for the most part mirrored levels of phosphorylated Akt, with little to no levels of phosphorylated p70S6k in the MDA-MB-435 cells and higher levels in the MDA-MB-468 cells. The MCF-7 cells had high levels of phosphorylated p70S6 kinase, but this correlated with high levels of total p70S6 kinase, as has been noted by others. These data are in agreement with findings by Lu et al. [6], and were used to establish the PTEN and Akt status of our particular cells.

We next examined the growth inhibition response of these cells exposed to the mTOR inhibitor rapamycin, the PI3K inhibitor LY294002, as well as the inhibitor to the mitogen-activated kinase (MAP-kinase) PD98059 (Figure 1B). Others have demonstrated that response to a rapamycin analog, CCI-779, currently in clinical trials, correlates with PTEN status [13, 14, 21]. The MDA-MB-435 cells, which express lower levels of activated Akt, demonstrated statistically significant less sensitivity to treatment with increasing concentrations of rapamycin (from 0 to 40 nM) compared to that displayed by the MCF-7 and MDA-MB-468 cells \(P < 0.05\). In concordance with our hypothesis that the observed effect is a result of inhibition of the PI3K/Akt signaling pathway in these cell lines, both the MCF-7 and MDA-MB-468 cells were susceptible to the LY294002 while the MDA-MB-435 cells were significantly less affected \(P < 0.05\). This is in contrast to the results obtained with the MAP kinase inhibitor PD98059. Little to no growth inhibition was observed in either

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**Figure 1.** Akt/PTEN pathway status correlates with growth inhibition response to treatment with PI3K inhibitors. (A) Western blot analysis of PTEN, phosphorylated and total Akt, phosphorylated and total p70S6k and actin expression levels in extracts from MDA-MB-435, MCF-7 and MDA-MB-468 cells serum starved for 18 h and then exposed for 0, 15, 30 and 60 min to 10% FBS. (B) MCF-7 (diamond), MDA-MB-435 (square) and MDA-MB-468 (triangle) cells were exposed continuously for 96 h to increasing concentrations of rapamycin (upper panel), LY294002 (middle panel) or PD98059 (lower panel). Growth was assessed by MTT dye conversion and presented as the percentage of control cell growth inhibition obtained from vehicle-treated cells grown in the same culture plate at 96 h. The data presented are the average of six replicate experiments.
the MDA-MB-468 or MCF-7 cells at concentrations up to 100 μM; growth inhibition was observed in the MDA-MB-435 cells. The MDA-MB-435 cells, which displayed very low levels of activated Akt, never reached an IC50 with either the rapamycin or the LY294002. The MCF-7 cells also did not reach an IC50 with rapamycin, but did with the LY294002. The MCF-7 cells did not reach an IC50 with LY294002, since the IC50 for the MCF-7 and the MDA-MB-468 cells, which displayed very low levels of activated Akt, never reached an IC50 with either the rapamycin or the LY294002. The MCF-7 cells also did not reach an IC50 with rapamycin, but did with the LY294002. The MDA-MB-468 cells did display heightened sensitivity to rapamycin, with an IC50 of 16.33 nM (0.577 SD). Interestingly, this was specific for inhibitors of the PI3K pathway, since the IC50 for the MCF-7 and the MDA-MB-468 cells of the MAPK inhibitor, PD98059, was never reached, while the MDA-MB-435 cells had an IC50 for this compound of 47 μM (5.6 SD).

**PTEN levels modify the response to treatment with PI3K/Akt pathway inhibitors**

Because there are many biological differences between the breast cancer cell lines used in these studies in addition to the differences observed in expression levels of PTEN that could affect their response to signal transduction inhibitors, we genetically manipulated the existing breast cancer cell lines to generate syngenic pairs of cell lines differing only in their PTEN status (Figure 2). In these experiments, the PTEN-positive MCF-7 cell line was transfected with either an empty expression vector (MCF-7 control) or an antisense PTEN to develop MCF-7 cell lines with greatly reduced PTEN expression (MCF-7 AS PTEN clone 3 and MCF-7 AS PTEN clone 9). We used the MCF-7 cell line because these more closely reflect the ERα-positive, wild-type p53 status reflected in clinical samples. Several clones were selected based upon inhibition of PTEN expression levels. The data presented in Figures 2, 3 and 4 using AS PTEN clones 3 and 9 are representative of experiments done with several of the clones. In these antisense PTEN cells, Akt became constitutively phosphorylated and displayed heightened response to treatment with 10% FBS. In both cases, total Akt protein was not affected. Downstream of Akt, phosphorylated levels of p70s6k also increased, though not as dramatically as the Akt. These results demonstrated that inhibition of PTEN expression directly affected both the constitutive as well as the inducible phosphorylation status of key downstream targets.

Since response to rapamycin and LY294002 treatment in the three breast cancer cell lines correlated with PTEN levels, we determined the growth response of the antisense PTEN cells to these compounds compared to the control cells. Exposure of the cells to 20 nM rapamycin resulted in decreasing the levels of phosphorylated p70S6 kinase in the AS PTEN MCF7 cells to those observed in the control MCF-7 cells (Figure 3A). As shown in the graph in Figure 3C, inhibition of PTEN expression correlated with increased sensitivity to the growth inhibitory effects of both rapamycin (upper panel) and LY294002 (lower panel). This is detailed in the table in Figure 3B. The rapamycin IC50s for the AS PTEN clones 3 and 9 were now reached at 13.7 and 28.9 nM, respectively, while the IC50s of the AS clones for LY294002 were decreased by 54% (3.2 μM) and 89% (0.8 μM), respectively, compared to that of the MCF-7 control cells (7.0 μM). These differences were statistically significant. Despite clone 9 having higher levels of PTEN, it was more susceptible to the antiproliferative effects of LY294002. While the reasons for this observation are not clear it indicates that the relationship between PTEN expression and susceptibility is not as robust for inhibitors of PI3K versus mTOR inhibitors.

**Genetic manipulation of PTEN expression levels results in altered cell cycle distributions in response to PI3 kinase pathway inhibitors**

Since the PTEN/Akt pathway has previously been described as important for cell proliferation [22], we next investigated what proliferative changes would be induced by alteration of PTEN expression levels (Figure 4). Flow cytometry analysis of these cells indicated a higher S-fraction in the AS PTEN 3 MCF-7 cells compared to both the control MCF-7 cells and the AS PTEN 9 cells (54% compared to 48%, P ≤ 0.04). While the differences are not dramatic, even small differences over a short period of time in vitro could result in much more pronounced long-term, in vivo differences. In response to exposure to 20 nM rapamycin, 55% of the control MCF-7 cells compared to 63% of the AS PTEN 3 and 67% of the AS PTEN 9 cells were found to be in the G1 + G2 phase. Exposure of the cells to 5 μM LY294002 inhibited the cell cycle inhibition in the antisense PTEN cells (87% and 91% for clones 3 and 9, respectively), with only 67% of the MCF-7 control cells found in G1 and G2 phases at the same concentration.
The results from these studies have several clinical as well as biological implications. In addition to constitutive activation of Akt, prior studies have indicated that deletions and/or mutations in the PTEN tumor suppressor gene result in a substantial number of intracellular anomalies, such as aberrant apoptotic responses, decreased cell adhesion, dysfunctional cell cycle regulation, and improper signal transduction [reviewed in 23], offering possibilities for the development of therapeutic strategies. In this current study, using both established breast cancer cell lines as well as our PTEN antisense MCF-7 cells, we have demonstrated that PTEN expression strongly correlates with cellular response to exposure to PI3 kinase pathway inhibitors. Suppression of PTEN expression resulted in dramatic increases in Akt phosphorylation. These data agree with recent findings by Gera et al. [24], which demonstrated that Akt activity determines sensitivity to mTOR inhibitors. The critical role of Akt activation in cell proliferation and survival and the potential of this pathway for therapeutic interventions have recently been reinforced by studies in NSCLC cell lines [10]. NSCLC frequently has constitutive activation of Akt. Treatment of these cells with
the PI3K inhibitor LY294002 induced apoptosis in cell lines with constitutive activation of the Akt and increased the susceptibility of these cell lines to chemotherapy and radiation therapy. An additional important observation is the similarities in the growth inhibitory effects between the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin, indicating that mTOR is indeed a relevant downstream element in the PI3K/Akt signaling pathway, as previously reported [25]. This concept is reinforced by recent data indicating that PTEN-negative malignant human cells and murine cells as well as spontaneous cancerous proliferations in PTEN heterozygous mice have increased activity of p70S6 kinase, a well defined downstream mediator of mTOR, which is reduced by CCI-779, an ester of rapamycin [13, 14]. Additional studies are ongoing to further define the functions of PTEN and its downstream target, Akt, in the cell cycle regulation and/or apoptosis responses observed upon inhibition of the PI3K and mTOR in these cell lines.

Importantly, from a clinical perspective, our proliferation data, in which our PTEN antisense MCF-7 cell lines were growth inhibited by both LY294002 and rapamycin to a much greater degree than our PTEN-positive control cells, indicate that PTEN-negative breast cancer tumors may be more sensitive to the antiproliferative effects of PI3K inhibition. Similar results have been observed by other groups in different cancer models [13, 14], as well as in the breast in response to mTOR inhibition [21], indicating that this is probably a general, tumor type-independent, observation. Furthermore, introduction of a constitutive active Akt into PTEN-negative prostate cancer cells further increased the susceptibility of these cells to CCI-779 [13]. In addition, treatment of PTEN heterozygous mice with CCI-779 resulted in decreased tumor size and neoplastic proliferation [14]. Overall, these data indicate that inhibition of the PI3 kinase pathway has the potential to be active in this aggressive tumor type and that this may be a biologically-related, tissue-type independent finding. Rapamycin analog compounds are already in clinical development for the treatment of patients with cancer [18]. CCI-779 has already completed phase I evaluation in patients with advanced cancer and is currently in a broad phase II developmental phase. The agent has demonstrated significant antitumor activity in patients with various tumor types such as breast cancer, non small cell lung cancer, soft tissue sarcomas and renal cell cancer among others. We have used rapamycin for in vitro studies and CCI-779 for in vivo studies and have obtained similar results with the two compounds [26], indicating that our findings with rapamycin would translate to results that could be anticipated with CCI-779 or the Novartis compound, RAD001.

The most relevant question based on the data presented in this paper, together with recent published information, is how to translate these observations to the clinic. At least two distinct strategies are possible for the development of clinical trials. First, the use of PTEN mutations or Akt activation as markers for upfront selection of patients. The second strategy will consist of not selecting patients for trial participation but evaluating the presence of PTEN mutations/Akt activation in a retrospective way to determine if a correlation exists between these molecular markers and indices of activity that will provide the rationale to explore in prospective clinical investigations the activity of CCI-779 in patients with Akt-hyperactive/PTEN-defective tumors.

In summary, constitutive signaling through the PI3K/Akt pathway in PTEN-defective tumors results in a strong dependence upon this pathway for growth and survival that can be exploited therapeutically. The data from this study indicate that tumors with PTEN mutations that result in higher Akt phosphorylation are more susceptible to the antiproliferative effects of inhibition of the PI3K/Akt pathway. Collectively, these data indicate that inhibition of PI3K/Akt signaling could be a potential strategy to treat patients with PTEN-negative tumors.

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