High Raf-1 Kinase Activity Protects Human Tumor Cells against Paclitaxel-induced Cytotoxicity

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Abstract

Paclitaxel (Taxol™) is becoming increasingly important in the treatment of many tumors, although a large proportion of tumors fail to respond to this drug. The identification of the processes that confer cellular paclitaxel resistance could provide potential targets for novel therapies that may help to eliminate paclitaxel-resistant tumors. Recent reports suggest that the Raf-1 protein kinase may have a profound influence on the level of paclitaxel-induced apoptosis. We have critically evaluated the relationship between Raf-1 kinase activity and de novo paclitaxel resistance in early-passage human cervical tumors. In the 12 cell lines studied, Raf-1 kinase activity was inversely correlated (P = 0.0016) with the level of cytoxicity induced by 60 nM paclitaxel. The relationship between these two parameters seems to be more than an epiphenomenon, because genetic down-regulation of Raf-1 kinase activity led to an approximately 4-fold increase in paclitaxel-induced cytotoxicity. The data from both our transfection studies and those on the 12 unper- turbed cell lines are consistent with Raf-1 kinase being a negative determinant of paclitaxel-induced cytotoxicity. Because the cytotoxicity of paclitaxel is primarily attributable to apoptosis, these data suggest that Raf-1 kinase acts to suppress paclitaxel-induced apoptosis. These data suggest that the clinical effectiveness of paclitaxel could be substantially improved by the use of Raf-1 kinase inhibitors, provided that a similar relationship between Raf-1 kinase activity and paclitaxel cytotoxicity exists in the clinic, especially in those tumor sites where paclitaxel is the current treatment of choice e.g., ovarian and breast cancer.

Introduction

Systemic chemotherapy after the appropriate surgical procedure is first-line treatment for many advanced tumors; however, the 5-year survival rates of patients with such tumors remain unacceptably low. One of the more effective drugs currently in clinical use is paclitaxel (Taxol™). Although this drug has enhanced the response rates of patients with advanced ovarian and breast cancer, the majority of these tumors are still not eliminated by this drug. The identification of the processes that confer paclitaxel resistance might lead to the development of strategies that further improve patient survival rates. Such information may also provide potential targets for gene therapy (once it has been sufficiently refined to allow the selective targeting of tumor cells) or might facilitate the development of new drugs to enhance paclitaxel cytotoxicity.

The relative toxicity of the various taxane compounds is correlated with the relative ability of these compounds to stabilize the microtubules (1). Paclitaxel increases microtubule stability by preventing tubulin depolymerization, which eventually leads to tubulin bundling (2, 3). These cytoskeletal changes lead to the induction of cell cycle arrest and cell death (4–6), with cells undergoing apoptosis within 20 h of paclitaxel exposure (7–9). The level of paclitaxel-induced apoptosis seems to have some dependency on Raf-1 kinase activity. Prior exposure of human tumor cells to the drug geldanamycin has been reported to collaterally diminish Raf-1 kinase activity and paclitaxel-induced apoptosis (10). The Raf-1 dependency of paclitaxel-induced apoptosis has, to date, only been demonstrated after the pharmacological inhibition of Raf-1 by geldanamycin in MCF7 breast cancer cells (10). However, geldanamycin seems to be relatively nonspecific and affects several cellular proteins (11–13), including the mutated p53 protein (14) that seems to be a negative prognostic factor for paclitaxel responsiveness in lung cancer patients (15). Moreover, whereas it is true that paclitaxel-induced tubulin polymerization does lead to the activation of (among others) the Raf-1 protein (10, 16), the biological consequences of this activation on the induction of apoptosis are unclear. Recent reports suggest that Bcl-2 targets the Raf-1 protein to the mitochondrial membranes, where this kinase phosphorylates (and thus inactivates) the proapoptotic Bad protein (17). However, Raf-1 located at the plasma membrane phosphorylates mitogen-activated protein kinase, which in turn phosphorylates Bcl-2 (10), thereby disrupting the association between Bcl-2 and the proapoptotic Bax protein (18), which then induces apoptosis. Thus, depending on the subcellular localization of the phosphorylated Raf-1 (and the relative levels of the pro- and antiapoptotic proteins), the paclitaxel-induced elevation of Raf-1 kinase activity could lead to either high or low levels of apoptosis. We reasoned that further characterization of the Raf-1 dependency of paclitaxel cytotoxicity in human tumor cells was required.

We thus determined the Raf-1 kinase dependency of paclitaxel cytotoxicity in 12 early-passage human tumor cell lines that we already knew exhibited a range of Raf-1 kinase activity. A second, although equally important, aspect of these studies was to establish the impact that genetic down-regulation of Raf-1 kinase activity had on cellular paclitaxel resistance. This
Materials and Methods

Human Cervical Tumor Cell Lines. The clonal selection techniques used to isolate the human cervical tumor cell lines used in this study have been published previously (19, 20). Briefly, approximately 10–12 single-cell-derived clones were established from each of 5 early-passage cell lines that had been frozen down after only 3 passages from being established from pretreatment punch biopsy samples (21). Twelve cloned cell lines were chosen that exhibited a range of Raf-1 kinase activity. After recovery from cryopreservation, the cell lines were maintained as monolayer cultures in DMEM:F12 media supplemented with 15% FCS (Life Technologies, Inc., Grand Island, NY) and antibiotics for three passages (subcultured every 4–5 days to ensure exponential growth) before the paclitaxel sensitivity of the cells was assessed simultaneously with the preparation of cellular protein extracts for Raf-1 kinase activity assays.

Clonogenic Cell Survival Assays. Two days before use, cells from exponentially growing stock cultures were detached using 0.25% trypsin and 1 mM EDTA at 37°C and placed into fresh 75-cm² tissue culture flasks. On the day of the experiment, cells from exponentially growing stock cultures were detached using 0.25% trypsin and 1 mM EDTA at 37°C. The cell pellet was then washed twice with warm PBS and resuspended in the DMEM:F12 media supplemented with 15% FCS (Life Technologies, Inc.) and antibiotics. Cells were seeded at densities of between 10² and 5 X 10⁶ cells/60-mm tissue culture dish. The Petri dishes were incubated for 6 h at 37°C (5% CO₂/95% air) to allow for cell attachment and then exposed to graded doses of paclitaxel (0–120 nm) for 24 h at 37°C (5% CO₂/95% air). Each paclitaxel survival curve consisted of a minimum of four drug concentration points. Each dose point was assayed by at least three separate experiments, with each assay consisting of three replicate plates. The results presented in this study represent the pooled data from a minimum of three experiments. After paclitaxel exposure, the drug-containing media were removed, and fresh media were added before being placed in the incubator. After 15 days at 37°C (5% CO₂/95% air), the colonies were fixed in 70% ethanol and stained with 10% methylene blue, and those colonies containing more than 50 cells were counted.

Data Handling and Presentation. The experimental data were fitted to the linear quadratic equation:

\[ -\ln SF = \alpha C + \beta C^2 \]

where SF is equivalent to the surviving fraction at a given drug concentration, C, and α and β are constants. The data were fitted to a linear quadratic function using the nonlinear regression PRIZM software package (Graphpad Software Inc., San Diego, CA). The fraction of cells surviving a clinically relevant paclitaxel concentration, i.e., 60 nm (22, 23), was calculated by substituting the derived constants and the appropriate dose point into the linear quadratic equation.

Transfection Procedure. We determined the effect that genetic manipulation of Raf-1 kinase activity had on paclitaxel-induced cytotoxicity in the HT8106/6 and HT212/9 cell lines. The full-length sense and antisense RAF1 constructs were created by ligating the EcoRI-EcoRI fragment of the p627 clone (American Type Culture Collection) into the pcDNA3 constitutive expression vector. This construct was then used to transform DH5α bacterial cells, and after 24 h, the bacterial colonies that contained recombinant plasmid were identified by colony hybridization using a radiolabeled RAF1 probe. Restriction endonuclease mapping was used to determine the orientation of the RAF1 gene in the positive colonies. Those colonies that had incorporated the RAF1 construct in the antisense or sense orientations were expanded, and the plasmids were purified. The antisense RAF1-pcDNA3 construct was designated pcDNA3-RAFJS, whereas the sense RAF1-pcDNA3 construct was designated pcDNA3-RAFTS. The pcDNA3, pcDNA3-RAFTS, and pcDNA3-RAFTJS constructs were electroporated into the HT180/6 and HT212/9 cell lines using the Bio-Rad gene pulser (Bio-Rad, Mississauga, Ontario, Canada) using the following procedure: (a) exponentially growing cells were detached by trypsin; (b) FCS was added to neutralize the trypsin; and (c) the cells were pelleted. The cells were then resuspended in serum-free DMEM:F12 media at 1 X 10⁶ cells/ml, placed into a Bio-Rad gene pulser cuvette, mixed with 25 μg of the appropriate linearized plasmid construct, and shocked at 275 V (pulse time, 5–30ms). After electroporation, the cells were diluted 10-fold with complete media and incubated at 37°C (5% CO₂/95% air) for 48 h. The media were then replaced with fresh media containing 700 μg/ml Geneticin (G418) to select the stably transfected clones. Every 2–3 days, fresh media containing G418 were added to the cultures for a period of approximately 4 weeks, at which time G418-resistant colonies were selected at random for clonal expansion. After the cells reached confluence, the cultures were subcultured, and the paclitaxel sensitivity and Raf-1 kinase activity were determined once they had reached exponential growth.

Determination of Raf Kinase Activity. In light of the finding that an 8-bp deletion can alter Raf-1 activity (24), we chose not to simply determine the level of phosphorylated 74-kDa Raf-1 protein but rather to directly determine Raf-1 kinase activity. Cell monolayers were detached by trypsinization, and the cells were washed twice in cold sterile PBS and adjusted to a concentration of 1 X 10⁶/ml, and 5 X 10⁶ cells were then pelleted and lysed in 1 ml of buffer A [50 mM Tris-Cl (pH 7.4), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, and 0.1% NP40] supplemented with 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. After incubation on ice for 60 min, the samples were centrifuged at 10,000 X g for 10 min at 4°C, and the supernatant was recovered. The cell lysate was then incubated with 5 μl of anti-Raf-1 antibody [500 μg of IgG0.25 ml of agarose conjugate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)] overnight at 4°C. In the initial stages of this study, we determined that the Raf-1 kinase activity of the immunoprecipitated Raf-1 protein from the HT212/3 cell extract rose when the anti-Raf-1 antibody level was increased from 1 to 3 μl, but that 3 and 5 μl of anti-Raf-1 antibody yielded essentially the same level of Raf-1 kinase activity. We elected to use the higher level of anti-Raf-1 antibody to ensure that the
antibody was always in excess. The immune complex was pelleted by centrifugation, washed once in buffer B (20 mM HEPES (pH 7.5), 40 mM EGTA, 20 mM β-glycerophosphate, 2 mM MgCl₂, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), and resuspended in 200 μl of buffer B. The level of Raf-1 kinase activity in 10 μl of this suspension (equivalent to 2.5 × 10⁵ cells) was determined using the Pierce colorimetric PKC³ assay kit Spinzyme Format (Pierce, Rockford, IL) according to the manufacturer's instructions. Briefly, 10 μl of the immunoprecipitate suspension were added to 5 μl of reaction buffer (2 mM ATP, 10 mM MgCl₂, 0.1 mM CaCl₂, 0.002% Triton X-100, 20 mM Tris (hydroxymethyl)-amino methane (pH 7.4)), and 5 μl (17.4 μg) of fluorescence-tagged myelin basic protein substrate. After incubation at 30°C for 30 min, 20 μl of the reaction mixture were placed onto the Spinzyme columns (which contain a membrane that preferentially binds phosphorylated proteins) and centrifuged at 2,000 × g for 1 min. After two washes with 250 μl of phosphopeptide-binding buffer (0.1 mM sodium acetate, 0.5 mM sodium chloride, and 0.02% sodium azide (pH 5.0)), the phosphorylated myelin basic protein substrate was recovered using 500 μl (two washes with 250 μl) of eluting buffer (0.1 M ammonium bicarbonate and 0.02% sodium azide (pH 8.0)) that was then added to 1 ml of distilled water, and the fluorescence intensity (excitation at 573 nm, emission at 589 nm, both with slit-widths of 5 nm) was determined by fluorescence spectroscopy. Graded amounts of purified PKC were used to construct a standard curve of the amount of phosphorylated substrate generated per IU of kinase activity simultaneously with the Raf-1 kinase determinations.

**Results**

The twelve cervical tumor cell lines studied exhibited a range of sensitivities to paclitaxel. There was a 3-fold variation in paclitaxel sensitivity between these 12 cell lines, with SF₅₀ values (the fraction of cells surviving exposure to 60 nM paclitaxel) ranging from 27–85%. These cell lines exhibited a 2-fold variation in Raf-1 kinase activity, with Raf-1 kinase activity levels ranging from 2.9–5.8 × 10⁻³ IU/10⁶ cells. Cell lines with low endogenous Raf-1 kinase activity were more sensitive to paclitaxel than those cell lines that had higher Raf-1 kinase activity. Linear regression analysis of these data revealed that the level of cytotoxicity induced by 60 nM paclitaxel was inversely correlated (P < 0.0016) to the endogenous Raf-1 kinase activity of the cell lines studied (Fig. 1).

To verify that the association between high Raf-1 kinase activity and paclitaxel resistance was not an epiphenomenon, we determined the impact that genetic down-regulation of Raf-1 kinase activity had on paclitaxel-induced cytotoxicity. Based on the linear regression analysis (Fig. 1), it would seem that an approximately 2-fold diminution of Raf-1 kinase activity would lead to a 2-fold enhancement in paclitaxel cytotoxicity in the tumor cell lines studied. Transfection with the pcDNA3 vector alone did not lead to any significant alterations in Raf-1 kinase activity in either the HT180/6 or HT212/9 cell line. Transfection with the RAF1 sense construct did not significantly alter either paclitaxel-induced cytotoxicity of anti-Raf-1 kinase activity in the HT180/6 cell line but led to a small increase in both Raf-1 kinase activity and paclitaxel resistance in the HT212/9 cell line (Fig. 2). Raf-1 kinase activity was down-regulated by 4- and 16-fold, respectively, in HT180/6 and HT212/9 cells that had been stably transfected with the RAF1 antisense construct (Fig. 2). The level of paclitaxel-induced cytotoxicity was highly dependent on Raf-1 kinase activity in the tumor cell lines studied. The genetic down-regulation of Raf-1 kinase activity led to a 2.5–4-fold increase in the cytotoxicity induced by 80 nm paclitaxel in HT180/6 and HT212/9 cells, respectively (Fig. 3). The relationship between Raf-1 kinase activity and paclitaxel sensitivity in the various RAF1 transfectants was essentially the same as that observed in the 12 unperturbed cell lines (Fig. 1).

**Discussion**

Although paclitaxel is one of the most effective chemotherapeutic agents available to treat solid tumors, a large proportion of such tumors are not cured by this drug. The identification of the processes that determine the cellular response to paclitaxel could lead to the development of novel therapeutic approaches that maximize the effectiveness of paclitaxel cytotoxicity. In many tumor cells, apoptosis seems to be the major

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³ The abbreviations used are: PKC, protein kinase C; HPV, human papillomavirus.
mode of cell death induced by paclitaxel (7–9). Recent reports suggest that the Raf-1 protein kinase may have a profound influence on the level of paclitaxel-induced apoptosis (10), although there is no definitive information on whether such changes also translate to similar changes in clonogenic survival. The purpose of these studies was to determine the Raf-1 kinase dependency of paclitaxel-induced cytotoxicity in human tumor cells.

The data presented in this study suggest that Raf-1 kinase activity is a major determinant of de novo paclitaxel resistance in human cervical cancer cells (Figs. 1–3). Genetic down-regulation of Raf-1 kinase activity leads to an approximately 4-fold increase in the cytotoxicity induced by 80 nM paclitaxel.

Interestingly, the genetic down-regulation of Raf-1 activity in cervical tumor cells leads to a completely different effect on paclitaxel cytotoxicity than when geldanamycin was used to inhibit Raf-1 in MCF7 breast cancer cells (10). One possible explanation for these discrepancies may lie in the fact that geldanamycin affects may other cellular proteins in addition to Raf-1 (11–13, 25). This lack of specificity is especially impor-
tant, because there are reports that PKC may play a role in mediating paclitaxel-induced cytotoxicity (26, 27). The data from both our transfection studies and from those on the 12 unperturbed cell lines are consistent with Raf-1 kinase being a negative determinant of paclitaxel-induced cytotoxicity. Because the cytotoxicity of paclitaxel is primarily attributable to apoptosis, these data suggest that Raf-1 kinase acts to suppress paclitaxel-induced apoptosis. Recent reports suggest that Bcl-2 targets Raf-1 to the mitochondrial membranes, where this kinase phosphorylates (and thus inactivates) the proapoptotic Bax protein (17).

Our demonstration that Raf-1 kinase activity is a major determinant of paclitaxel cytotoxicity in human cervical tumors cells is an exciting observation. The manipulation of this pathway could potentially lead to an increase in the therapeutic effectiveness of paclitaxel. Many biotechnology companies are already developing inhibitors that specifically target the Raf-1 pathway. It might thus be possible to rapidly translate these laboratory studies into tangible benefits for patients if a similar relationship between Raf-1 kinase activity and paclitaxel cytotoxicity exists in the clinic, especially in those tumor sites where paclitaxel is the treatment of choice, e.g., ovarian and breast cancer. Our data (Fig. 3) suggest that a complete inhibition of Raf-1 kinase activity is not essential for a clinically meaningful (e.g., 2-fold) sensitization to paclitaxel. Although it would be of interest to determine whether a similar relationship between Raf-1 kinase activity and paclitaxel cytotoxicity exists in bone marrow stem cells (the principal dose-limiting normal tissue for paclitaxel), this information may be of academic interest, because autologous peripheral blood stem-cell support is increasingly an integral part of the treatment regimen for patients receiving high-dose paclitaxel therapy (28). However, a detailed knowledge of the Raf-1 dependency of paclitaxel cytotoxicity in ovarian or breast cancer cells is essential to establish the feasibility of using Raf-1 inhibitors to enhance paclitaxel cytotoxicity in these sites. Whereas our preliminary data have established that Raf-1 kinase activity is a major determinant of paclitaxel resistance in human cervical tumor cell lines, it is unclear whether these findings can be extrapolated to epithelial ovarian cancer. One reason that might prevent such extrapolations is that p53 status has been implicated as a determinant of paclitaxel cytotoxicity (15, 29), coupled with the fact that cervical tumor cells are generally infected with HPV16 and/or HPV18 and may thus have an altered p53 function. However, recent reports suggest that (unlike the situation in which HPV16 is overexpressed in transfection studies) p53 functionality may not be completely abrogated by the HPV16-E6 protein in situations such as cervical cancer, where the persistence of the HPV-infected host cells is necessary (30). Whether or not p53 is down-regulated in cervical cancer cells may be an unwarranted concern, because a number of studies have shown that p53 status may have little impact on the level of paclitaxel-induced apoptosis (31, 32), although it should be noted that the presence of a mutated p53 protein seems to be a negative prognostic factor for paclitaxel responsiveness in lung cancer patients (15).

In summary, these in vitro data indicate that Raf-1 kinase activity has a significant influence on the level of cell death induced by paclitaxel, and that 2–4-fold increases in paclitaxel-induced cytotoxicity can be induced when Raf-1 kinase activity levels are decreased. Although these data raise the possibility of using Raf-1 kinase inhibitors to significantly enhance tumor cell elimination by paclitaxel, considerable care is required in extrapolating these in vitro findings to the clinical setting. Further characterization of the relationship between Raf-1 kinase activity and paclitaxel responsiveness in the clinic and of those parameters that reduce the Raf-1 dependency of paclitaxel cytotoxicity is clearly needed before Raf-1 kinase inhibitors can be considered for clinical trials.

References
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