Regulation of Docetaxel-Induced Apoptosis of Human Melanoma Cells by Different Isoforms of Protein Kinase C

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Abstract
Our previous studies showed that docetaxel-induced apoptosis of human melanoma cells was dependent on the activation of the c-jun NH2-terminal kinase (JNK) signaling pathway but was inhibited by the extracellular signal–regulated kinase (ERK)-1/2 pathway. However, the mechanisms by which these pathways were modulated by docetaxel were not clear. We report here that docetaxel induces activation of protein kinase C (PKC) signaling differentially through PKCζ and PKCβ isoforms. Activation of PKCζ was most marked in docetaxel-resistant cells and paralleled the activation of the ERK1/2 pathway. Inhibition of PKCζ by small interfering RNA molecules resulted in down-regulation of phosphorylated ERK1/2 and sensitization of cells to docetaxel-induced apoptosis. Experiments also showed that β-tubulin class III, a molecular target of docetaxel, coimmunoprecipitated with PKCζ and colocalized in confocal microscopic studies. In contrast to PKCζ, high levels of activated PKCβ were associated with activation of the JNK pathway and sensitivity to docetaxel. Activation of PKCβ seemed to be upstream of JNK because inhibition of PKCβ by small interfering RNA abrogated activation of the JNK pathway. Although PKCβ could be activated in resistant cells, downstream activation of JNK and c-Jun did not occur. In summary, these results suggest that the outcome of docetaxel-induced apoptotic events in human melanoma cells depends on their PKC isoform content and signaling responses. PKCζ was associated with prosurvival signaling through ERK, whereas PKCβ was associated with proapoptotic responses through JNK activation. (Mol Cancer Res 2007;5(10):1073–81)

Introduction
The incidence of melanoma continues to increase in many parts of the world that have predominantly Caucasian populations. A prominent factor in the progression of human melanoma is the resistance to the conventional therapeutic modalities including radiotherapy, immunotherapy, and chemotherapy. A growing body of evidence suggests that resistance to therapy is largely due to resistance of melanoma cells to induction of apoptosis (1). A better understanding of the mechanisms involved in melanoma resistance to chemotherapy-induced apoptosis is thus required to improve the therapy of the disease.

Protein kinase C (PKC) consists of a family of intracellular structurally related serine/threonine kinases comprising at least 11 isoforms that play fundamental roles in cell proliferation, differentiation, and apoptosis (2, 3). They have been classified into “conventional” PKCs (α, β1, β2, and γ), which are activated by calcium or diacylglycerol; “novel” PKCs (δ, ε, η, μ, and θ), which are dependent on diacylglycerol but independent of calcium; and “atypical” PKCs (λ, ζ, and η), which are independent of calcium or diacylglycerol (2). The existence of several PKC isoforms with unique cofactor requirements, intracellular localization, and cellular or tissue distribution suggests a specialized role for each isoform in the control of cellular functions. PKCα, PKCβ, and PKCδ have been mainly associated with antiapoptotic effects in various systems (2, 4-6), whereas PKCδ, PKCθ, and PKCζ have been implicated as proapoptotic kinases (7, 8). The molecular mechanisms by which PKC isoforms regulate distinct pathways remains largely unknown. PKCs are activated in cells following stimulation with a wide variety of agonists including growth factors, antigens, cytokines, and neurotransmitters, as well as several anticancer agents (9-11). On activation, PKC isoforms may translocate to different subcellular sites where they access their substrates (12) such as members of the mitogen-activated protein kinase family (13-15).

Taxanes such as paclitaxel and docetaxel represent an important class of anticancer agents displaying efficacy against many cancers including a proportion of patients presenting with malignant melanoma. Initially described as antimitotic agents, taxanes bind to β-tubulins and stabilize the microtubular network, thus resulting in the block of the cell cycle at G2-M and subsequent apoptosis of the cells (16, 17). Previous studies have shown that taxanes may also inappropriately induce activation of prosurvival signaling pathways such as Ras-Raf-mitogen-activated protein kinase/extracellular signal–regulated
kinase (ERK) kinase-ERK, leading to cancer cell resistance (16, 17), and we have reported that inhibition of ERK1/2 sensitized melanoma cells to docetaxel-induced apoptosis (18). The mechanism of activation of the ERK1/2 pathway by taxanes, however, is unclear. Activation of ERK1/2 by TRAIL or by phorbol 12-myristate 13-acetate was dependent on activation of PKC (4, 14) but whether activation of the ERK1/2 pathway by taxanes also involves activation of PKC is not established. Recently, we have shown that docetaxel-induced apoptosis of melanoma cells was determined by activation of two opposing signaling pathways: the c-jun NH2-terminal kinase (JNK) and the ERK1/2 pathways (18). Melanoma sensitivity to docetaxel-induced killing was entirely dependent on activation of the JNK pathway and was inhibited by activation of the ERK1/2 pathway (18). In the present study, we provide evidence that ERK1/2 and JNK signaling pathways are modulated in response to docetaxel by the relative levels of activation of PKCζ and PKCδ. In addition, class III β-tubulin seemed to be physically associated with the PKCζ isoform whose activation was functionally associated with docetaxel resistance. These results add new insights into the resistance mechanisms of human melanoma to docetaxel.

Results

Docetaxel-Induced Activation of PKC Has Differential Effects on Apoptosis Induced in Different Melanoma Cell Lines

To study the role of PKC in docetaxel-induced apoptosis, the pan-PKC inhibitor GF109203X was used in the assays. IgR3 and MM200 cells that are inherently sensitive and resistant to docetaxel, respectively (18), were treated with GF109203X 1 h before adding docetaxel for another 48 h. Apoptosis was measured by the propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE. Among the PKC isoforms that have been implicated in the regulation of apoptosis, PKCζ is generally believed to be proapoptotic, whereas PKCδ is antiapoptotic (2, 4, 19, 20). The kinetics of activation of PKC isoforms, δ and ε, were therefore studied in IgR3 and MM200 cells before and after exposure to docetaxel. Figure 1B shows that docetaxel induced a gradual loss of PKCζ in IgR3 cells at 2 h following treatment but not in MM200 cells. PKCζ phosphorylation was robustly induced after treatment with docetaxel in MM200 cells whereas it was only weakly detected in IgR3 cells, although slight stimulation of PKCζ phosphorylation did occur and peaked at 16 h in these cells. Consistent with other reports (21, 22), the expression of the phosphorylated form of PKCζ correlated with the activation of JNK and its downstream gene c-Jun in IgR3 cells but not in MM200 cells.

The phosphorylated form of PKCζ was detected in IgR3 and MM200 cells under normal growth conditions and increased after treatment with docetaxel in both IgR3 and MM200 cells albeit with very different kinetics. Increased phosphorylated PKCζ peaked in IgR3 cells at 4 h whereas the response in MM200 cells was less and occurred at 16 to 24 h. Results also showed that expression of phosphorylated PKCζ correlated with the activation of JNK and its downstream gene c-Jun in IgR3 cells but not in MM200 cells.

FIGURE 1. Involvement of PKC signal transduction pathway in docetaxel-induced apoptosis. A. Effects of PKC inhibitor, GF109203X, on docetaxel-induced apoptosis of melanoma cells. IgR3 and MM200 cells were treated with or without GF109203X for 1 h before adding docetaxel at 20 nmol/L for another 48 h. Apoptosis was measured by the propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE. B. Activation of PKC isoforms by docetaxel. IgR3 and MM200 cells were treated with docetaxel at 20 nmol/L for the indicated time periods. Whole-cell lysates were subjected to Western blot analysis. Western blot analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels was included to show that equivalent amounts of protein were loaded in each lane. Representative of two or more individual experiments.
PKC Is Upstream of Activation of the ERK1/2 Pathway

PKC is known to activate ERK1/2 pathway by stimulating the Ras-Raf/mitogen-activated protein kinase/ERK kinase-ERK pathway (21, 22). To investigate whether activation of ERK1/2 in response to docetaxel treatment is downstream of PKC, we treated IgR3 and MM200 cells with the pan-PKC inhibitor GF109203X 1 h before adding docetaxel for another 16 h [TRAIL is known to induce ERK1/2 activation via PKC (4) and was used as a positive control]. MM200 harbor the active BRAF mutation V599E (V599EBRAF), whereas IgR3 carry the wild-type BRAF. Docetaxel-induced ERK1/2 phosphorylation was reduced in cells pretreated with GF109203X (Fig. 2A). In studies on a wider panel of melanoma cells, GF109203X also inhibited docetaxel-induced ERK1/2 activation in six of nine cell lines (Fig. 2B). The inhibitory effect of GF109203X was not seen in ME4405 and Mel-AT, which we have previously

FIGURE 2. Involvement of PKC signaling in docetaxel-induced activation of ERK1/2. A, IgR3 and MM200 cells were treated with or without GF109203X for 1 h before adding docetaxel at 20 nmol/L for another 8 h. Whole-cell lysates were subjected to Western blot analysis of ERK1/2. Representative of two individual experiments. B, Docetaxel-induced ERK1/2 is correlated with activation of PKC signaling pathway. Cells were treated with or without GF109203X for 1 h before adding docetaxel at 20 nmol/L for another 8 h before harvest. Whole-cell lysates were subjected to Western blot analysis. The relative expression of activated ERK1/2 was determined as described in Materials and Methods. Columns, mean of three individual experiments; bars, SE. C, Down-regulation of PKC expression inhibits docetaxel-induced activation of ERK1/2. IgR3 and MM200 cells were transfected with either a nontargeting siRNA (control siRNA) or with a PKCq-specific siRNA sequence (Dharmacon) at 50 and 100 nmol/L for 24 h. Twenty-four hours later, whole-cell lysates were subjected to Western blot analyses (top). MM200 and IgR3 cells were transfected with either a nontargeting siRNA (control siRNA) or a PKCq-specific siRNA sequence at 100 nmol/L for 24 h. Twenty-four hours later, transfected cells were treated with docetaxel at 20 nmol/L for another 8 h before harvest. Whole-cell lysates were subjected to Western blot analyses (bottom). Representative of two individual experiments. D, Down-regulation of PKCq expression sensitizes melanoma cells to docetaxel-induced apoptosis. MM200 and IgR3 cells were transfected with either a nontargeting siRNA (control siRNA) or with a PKCq-specific siRNA sequence at 100 nmol/L for 24 h. Twenty-four hours later, transfected cells were treated with docetaxel at 20 nmol/L for another 48 h. Apoptosis was measured by the propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE.
shown to have low or undetectable levels of PKCq (4). These results suggested that docetaxel-induced ERK1/2 activation occurred as a consequence of PKC activation, possibly through the PKCq.

To substantiate this notion, we studied the effect of inhibition of PKCq by using a specific small interfering RNA (siRNA) sequence against PKCq. IgR3 and MM200 cells were transfected with either a nontargeting siRNA (control siRNA) or with a PKCq-specific siRNA sequence at 50 and 100 nmol/L for 24 h. Twenty-four hours later, whole-cell lysates were subjected to Western blot analyses. Representative of two individual experiments. Down-regulation of PKCq expression inhibits docetaxel-induced JNK pathway activation. MM200 and IgR3 cells were transfected with either a nontargeting siRNA (control siRNA) or with a PKCq-specific siRNA sequence at 100 nmol/L for 24 h. Twenty-four hours later, transfected cells were treated with docetaxel at 20 nmol/L for another 3 h before harvest. Whole-cell lysates were subjected to Western blot analyses. Representative of two individual experiments.

Results shown in Fig. 2C and D indicated that inhibition of PKCq expression markedly inhibited docetaxel-induced ERK1/2 activation and sensitized melanoma cells to docetaxel-induced apoptosis, particularly MM200 cells (P < 0.01, two-tailed Student’s t test). Inhibition of PKCq also increased Bax activation and the mitochondrial membrane potential changes in cells treated with docetaxel (data not shown). These results provide further evidence that PKCq is acting upstream of docetaxel-induced ERK1/2 activation and that its inhibition sensitized melanoma cells to docetaxel-induced apoptosis.

**FIGURE 3.** PKCq is upstream of activation of the JNK pathway. A. Down-regulation of PKCq expression in IgR3 and MM200 cells using siRNA. IgR3 and MM200 cells were transfected with either a nontargeting siRNA (control siRNA) or with a PKCq-specific siRNA sequence at 50 and 100 nmol/L for 24 h. Twenty-four hours later, the whole-cell lysates were subjected to Western blot analyses. Representative of two individual experiments. B. Down-regulation of PKCq expression inhibits docetaxel-induced JNK pathway activation. MM200 and IgR3 cells were transfected with either a nontargeting siRNA (control siRNA) or with a PKCq-specific siRNA sequence at 100 nmol/L for 24 h. Twenty-four hours later, transfected cells were treated with docetaxel at 20 nmol/L for another 3 h before harvest. Whole-cell lysates were subjected to Western blot analyses. Representative of two individual experiments. C and D. Down-regulation of PKCq expression inhibits docetaxel-induced apoptotic events. MM200 and IgR3 cells were transfected with either a nontargeting siRNA (control siRNA) or with a PKCq-specific siRNA sequence at 100 nmol/L for 24 h. Twenty-four hours later, transfected cells were treated with docetaxel at 20 nmol/L for another 24 or 48 h before harvest. Cells treated for 48 h were analyzed for the sub-G1 DNA content using the propidium iodide method by flow cytometry (C). Conformational changes of Bax were measured by flow cytometry using a Bax NH2-terminal epitope-specific antibody that specifically recognizes the activated form of Bax. Changes in the mitochondrial membrane potential were measured by JC-1 using flow cytometry (D). Representative of three individual experiments. Columns, mean of three individual experiments; bars, SE.

**Docetaxel-Induced Activation of JNK Is Mediated by Activation of PKCq**

Previously, we have shown that docetaxel-induced apoptosis of melanoma cells is determined by the degree of activation of two opposing signaling pathways: the JNK and ERK1/2 pathways (18). Activation of the JNK pathway has been reported to lie downstream of different mediators including PKCq (11, 23, 24). To investigate whether PKCq functions as an upstream effector of the JNK in response to docetaxel treatment, a specific PKCq siRNA was used. IgR3 and MM200 cells were transfected with PKCq siRNA and the levels of PKCq and PKCq were analyzed by immunoblotting. Data shown in Fig. 3A revealed that with this approach, PKCq expression could be inhibited by 89% in IgR3 and 87% in MM200 cells whereas PKCq expression was not affected.

To study the effect of PKCq on the docetaxel-induced apoptotic machinery, IgR3 and MM200 cells transfected with PKCq siRNA were treated with docetaxel for 3 h before harvest and levels of activated JNK and c-Jun were analyzed by Western blot. The results presented in Fig. 3B indicate that for IgR3 cells, PKCq is required for activation of JNK and its
target, c-Jun, in that cells transfected with PKC\(\alpha\) siRNA were unable to activate JNK or c-Jun in response to docetaxel treatment. Whereas PKC\(\alpha\) activation did occur in MM200 cells treated with docetaxel, no c-Jun or JNK phosphorylation was observed. As a functional consequence, IgR3 cells transfected with PKC\(\alpha\) siRNA were also strongly prevented to undergo apoptosis in response to docetaxel (\(P < 0.01\), two tailed Student’s \(t\) test; Fig. 3C). Similar results were also obtained using the PKC\(\alpha\)-specific inhibitor Rottlerin (5 \(\mu\)mol/L; data not shown). Moreover, events downstream of JNK pathway activation such as activation of Bax and mitochondrial membrane potential changes were similarly affected in IgR3 cells by inhibition of PKC\(\alpha\) (Fig. 3D). In contrast, the ablation of the relatively low levels of PKC\(\alpha\) in the docetaxel-resistant MM200 cells had comparatively little effect on apoptosis levels, Bax, and mitochondrial membrane potential changes, which were all basal values in these assays.

**PKCs Is Associated with Class III \(\beta\)-Tubulin in Melanoma Cells**

We have reported elsewhere\(^3\) that high expression levels of class III \(\beta\)-tubulin led to melanoma cell resistance to docetaxel-induced apoptosis and that inhibition of its expression sensitized melanoma cells to docetaxel-induced killing. To understand the link between these events, we assessed the possible interaction between PKC\(\alpha\) and PKC\(\alpha\) with class III \(\beta\)-tubulin by immunoprecipitation studies from IgR3 and MM200 before and after treatment with docetaxel. Class III \(\beta\)-tubulin precipitates were analyzed by Western blot for PKC\(\alpha\) or PKC\(\alpha\) and class III \(\beta\)-tubulin. Figure 4A shows that there was a strong association between class III \(\beta\)-tubulin and PKC\(\alpha\) in MM200 cells before treatment with docetaxel, whereas after 3 h, PKC\(\alpha\) coprecipitation was barely detected. In comparison with MM200 cells, the association between class III \(\beta\)-tubulin and PKC\(\alpha\) in IgR3 was comparatively low and was not changed before and after treatment with docetaxel. The results also showed no association before and after treatment with docetaxel between class III \(\beta\)-tubulin and PKC\(\alpha\) in either cell line (Fig. 4A).

Dual-color confocal microscopy was then used to determine the subcellular localization of the two PKC isoforms in relation to class III \(\beta\)-tubulin. IgR3 and MM200 cells were fixed and immunostained with anti-\(\beta\)-tubulin class III monoclonal antibody and anti-PKC\(\alpha\) or anti-PKC\(\alpha\) polyclonal antibodies. This analysis showed that class III \(\beta\)-tubulin was distributed throughout the cytoplasm of both cell lines and its pattern of staining was mostly unchanged in treated cells apart from the phototypic changes that occur in response to docetaxel (flattening in MM200 cells and rounding in IgR3; Fig. 4B and C). The distribution of class III \(\beta\)-tubulin largely overlapped that of PKC\(\alpha\), which was observed throughout the cytoplasm in mainly punctate areas, and after treatment with docetaxel, a small proportion of the PKC\(\alpha\) shifted to peripheral regions of the cells, particularly in MM200 cells. The PKC\(\alpha\) isoform was distributed in a cytoplasmic pattern in both cell lines, with some staining also observed in the nuclear region of IgR3 cells after docetaxel treatment (Fig. 4C). Prominent localization of PKC\(\alpha\) was also seen in the periphery of untreated IgR3 but not in MM200 cells, although, in response to docetaxel, some PKC\(\alpha\) was redistributed to the cell periphery in MM200 cells.

**Discussion**

Our previous studies have suggested that sensitivity of melanoma cells to docetaxel is determined by the balance between activation of the JNK and ERK1/2 pathways. In the present study, we report that the relative activation of PKC\(\alpha\) and PKC\(\alpha\) isoforms by docetaxel seemed to determine the activation of these two pathways. PKC\(\alpha\) seemed to be upstream of activation of the ERK1/2 pathway whereas PKC\(\alpha\) induced activation of the JNK pathway. Knockdown studies of PKC\(\alpha\) by siRNA decreased activation of ERK1/2 and resulted in sensitization of melanoma cells to docetaxel-induced apoptosis. In contrast, inhibition of PKC\(\alpha\) expression resulted in nearly complete abrogation of the apoptotic events induced by docetaxel.

PKC\(\alpha\) cleavage and activation has been reported as a proapoptotic response to various apoptotic stimuli such as radiation, chemotherapeutic agents, and ligation of Fas and tumor necrosis factor-\(\alpha\) receptors (9-11), whereas the cleavage and activation of PKC\(\alpha\) has been mainly associated with inhibition of apoptosis (2, 4). The mechanism of activation of PKC\(\alpha\) by various proapoptotic stimuli involved a series of posttranslational modifications such as phosphorylation and cleavage (2). Although distinct tyrosine and serine/threonine phosphorylations can lead to an antiapoptotic or proapoptotic response, cleavage of PKC\(\alpha\) to its 40-kDa, fully active catalytic fragment was reported to be the only definitive modification that leads to a proapoptotic response (25). Results in the present study revealed that both cleavage and phosphorylation of PKC\(\alpha\) were induced in docetaxel-sensitive IgR3 cells following treatment with docetaxel, indicating that both might be of significance in melanoma sensitivity to docetaxel. In our previous studies, we found that docetaxel-induced apoptosis is entirely dependent on activation of the JNK pathway; however, the mechanism of this activation was not determined.

The findings in this study showing inhibition of docetaxel-induced apoptosis by a specific PKC\(\alpha\) siRNA support a key role for PKC\(\alpha\) activation in docetaxel-induced apoptosis. Docetaxel-induced phosphorylation of c-Jun, a known downstream target of JNK, was inhibited in cells transfected with PKC\(\alpha\) siRNA, showing that PKC\(\alpha\) is upstream of JNK pathway activation. The mechanism(s) by which PKC\(\alpha\) activates the JNK pathway is still unclear but PKC\(\alpha\) was reported to activate the MKK7-JNK-activator protein-1 pathway (11, 23). We saw evidence for redistribution of some PKC\(\alpha\) to the nucleus of IgR3 cells following docetaxel treatment, placing it in proximity to JNK and its target c-Jun. Other reports revealed that PKC might induce activation of the JNK through the receptor for activated C kinase 1 (26). PKC\(\alpha\) was also localized prominently in the cell margins of IgR3 cells where receptor for activated C kinase

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\(^3\) Mhaidat N, Thorne R, Zhang XD, and Hersey P. Melanoma cell sensitivity to Docetaxel-induced apoptosis is determined by class III \(\beta\)-tubulin levels (submitted).
1 is expected to be found. Moreover, the translocation of activated PKC isoforms into different intracellular organelles including the endoplasmic reticulum may also contribute to the activation of mitogen-activated protein kinase signaling. More work therefore needs to be done to understand the events linking PKC\(\text{c}\) with JNK activation and especially to determine the apparent deficiency of this network in MM200 cells.

In contrast to PKC\(\text{c}\), activation of the PKC\(\text{q}\) isoform has been reported to be antiapoptotic in various cellular systems including lung cancer cells (27), T lymphocytes (28), and prostate cancer cells (29). Overexpression of PKC\(\text{q}\) inhibited apoptosis of melanoma (4), breast cancer (30), and glioma cells (2). In the current study, we found that the full-length PKC\(\text{q}\) undergoes gradual down-regulation in response to docetaxel treatment in the sensitive, but not the resistant, cells. Although docetaxel could induce PKC\(\text{c}\) phosphorylation in both these cell lines, this activation was particularly robust and sustained in the resistant MM200 cells. These results suggested that down-regulation and/or failure to activate PKC\(\text{c}\) might contribute to melanoma sensitivity to docetaxel-induced killing. Silencing of PKC\(\text{c}\) by siRNA, showing that inhibition of PKC\(\text{c}\) resulted in the sensitization of melanoma cells to docetaxel-induced apoptosis, supported this concept.

Previous studies have shown that activation of PKC isoforms leads to translocation to particulate fractions such as Golgi, endoplasmic reticulum, nucleus, and cytoskeleton

FIGURE 4. PKC\(\text{c}\) associated with class III \(\beta\)-tubulin in melanoma cells. A. Whole-cell lysates from IgR3 and MM200 cells with or without treatment with docetaxel at 20 nmol/L for 3 h were subjected to immunoprecipitation with class III \(\beta\)-tubulin polyclonal antibody. The resulting precipitates were analyzed by Western blotting for the presence of class III \(\beta\)-tubulin, PKC\(\text{c}\), and PKC\(\text{c}\). Equal volumes of the input lysates were used as protein detection controls. Representative of two experiments. B and C. IgR3 and MM200 cells were grown on coverslips 16 to 24 h before treatment with or without docetaxel at 20 nmol/L for 3 h. Cells were fixed with 3.8% formaldehyde and permeabilized (as indicated in Materials and Methods) before incubation with monoclonal antibodies against class III \(\beta\)-tubulin in combination with PKC\(\text{c}\) polyclonal antibody (B) or PKC\(\text{c}\) polyclonal antibody (C). Antibodies were decorated with either Alexa 488–conjugated secondary antibodies (mouse) or Alexa 594 (rabbit). Images were recorded using laser-scanning confocal microscopy at similar instrument settings.
(31-35) where they access different substrates such as members of the mitogen-activated protein kinase family (12, 13, 15). The coimmunoprecipitation studies reported here indicated that PKC\(\zeta\) was specifically colocalized with class III \(\beta\)-tubulin in the resistant MM200 cells and weakly in the sensitive IgR3 cells. This association, which occurred in the cytoplasmic fraction, was reduced following treatment with docetaxel. These results are consistent with the view that PKC\(\zeta\) activity might be able to dictate the distribution and incorporation of class III \(\beta\)-tubulin. Some of the PKC\(\zeta\) was redistributed to the cell periphery in MM200 cells as a result of docetaxel treatment. One defined outcome was the activation of ERK1/2 signaling, and this event may be associated with the redistribution of PKC\(\zeta\) following docetaxel treatment. Indeed, it has been reported that 20% to 40% of ERK1/2 is localized to the microtubules (17) and the redistribution of PKC\(\zeta\) may enable this activation to occur.

In summary, the results indicate that docetaxel-induced apoptosis of melanoma cells is modulated by activation of the PKC pathway; we have collated our findings in Fig. 5. Binding of docetaxel to microtubules induces their polymerization and activation of the PKC\(\zeta\) and PKC\(\epsilon\) isoforms. PKC\(\epsilon\) seemed to be upstream of activation of ERK1/2 and contributed to resistance of both melanoma cell lines to docetaxel-induced killing. This occurred by inhibition of Bax activation, down-regulation of Bim, up-regulation of Mcl-1, and inactivation of BAD by phosphorylation (18). In contrast to PKC\(\zeta\), activation of PKC\(\epsilon\) led to the activation of the JNK/c-Jun pathway. Activation of this pathway mediated docetaxel-induced apoptosis by phosphorylation and inactivation of Bcl-2, activation of caspase-2, and, consequently, activation of Bax that resulted in mitochondrial membrane perturbations and apoptosis (18). However, although PKC\(\epsilon\) was activated in both melanoma cell lines, activation of the JNK/c-Jun pathway was only seen in IgR3 and not MM200 cells. How then do the MM200 resist the activation of JNK when docetaxel can activate PKC\(\epsilon\) in these cells? Our ongoing studies will concentrate on understanding the process whereby PKC\(\epsilon\) signaling seems to be inactive or perhaps deactivated in docetaxel-resistant melanoma cells.

**Materials and Methods**

**Cell Lines**

Human melanoma cell lines Me4405, Me1007, IgR3, Mel-FH, Mel-RM, Mel-CV, Mel-AT, SK-mel-28, SK-mel-110, and MM200 have previously been described (36). MM200 and Sk-Mel-28 harbor the active BRAF mutation V599E (\(^{\text{V599E-BRAF}}\)), whereas the others carry the wild-type BRAF. The cell lines were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories).

**Antibodies and Other Reagents**

Docetaxel (Taxotere), kindly provided by Aventis Pharma SA, was stored as a 100 mmol/L solution in absolute ethanol at −80°C and diluted with the medium before use. The monoclonal antibody against phosphorylated ERK1/2 and the rabbit polyclonal antibodies anti-PKC\(\zeta\), PKC\(\epsilon\), and their phosphorylated forms were purchased from Santa Cruz Biotechnology. The rabbit polyclonal antibodies against JNK/ stress-activated protein kinase, ERK1/2, and phospho-c-Jun and the monoclonal antibody anti–phospho-JNK were purchased from Cell Signaling Technology. The rabbit polyclonal anti-Bax...
against amino acids 1 through 20 was purchased from Upstate Biotechnology. The 107.3 mouse immunoglobulin G1 monoclonal antibody was purchased from PharMingen, and rabbit immunoglobulin G from Sigma Chemical Co. The monoclonal and polyclonal anti–class III β-tubulin were purchased from Sigma-Aldrich. The PKC inhibitor bisindoylmaleimide I (GF109203X) and the PKCδ inhibitor Rottlerin were from Calbiochem.

Apoptosis
Quantitation of apoptotic cells by measurement of sub-G1 DNA content using the propidium iodide method was carried out as described elsewhere (36).

Indirect Immunofluorescence and Confocal Microscopy
Melanoma cells were seeded onto sterile glass coverslips in 24-well plates (Falcon 3047; Becton Dickinson) 16 to 24 h before treatment with or without docetaxel at 20 nmol/L for 3 h. Cells were then washed with PBS followed by fixation with 3.8% paraformaldehyde for 10 min. Cells were then permeabilized with 0.1% Triton X-100 in PBS.

Fixed cells were incubated for 30 min with PBS containing 3% bovine serum albumin to block nonspecific binding sites, then incubated for 1 h with the primary antibodies diluted in 1% bovine serum albumin at concentrations recommended by the manufacturer. Cells were washed with PBS and incubated with fluorochrome-conjugated secondary antibodies (Alexa 488 antimouse or Alexa 594 antirabbit at 1:400; Invitrogen). Control staining was also done using nonimmune rabbit or isotype-matched mouse immunoglobulins, secondary antibody alone controls, and samples where there was omission of either primary antibody. Coverslips were mounted in SlowFade Gold (Invitrogen) and examined with a Zeiss confocal LSM510 microscope.

Flow Cytometry
Immunostaining on intact and permeabilized cells was carried out as previously described (37). Analysis was carried out with a Becton Dickinson FACScan flow cytometer. The percentage of antigen-positive cells was calculated as the difference in positive area between the positive and negative control histograms. The positive area was that to the right of the intersection of the two curves (36).

Mitochondrial Membrane Potential ($\Delta \Psi_m$)
Tumor cells were seeded at $1 \times 10^5$ per well in 24-well plates and allowed to reach exponential growth for 24 h before treatment. JC-1 staining was done according to the manufacturer’s instructions (Molecular Probes). Briefly, adherent cells and nonadherent cells were collected and washed with PBS. Cells were then incubated with 10 μg/mL JC-1 in warm PBS at 37°C for 15 min. After washing with PBS, the cells were analyzed with a FACScan flow cytometer (Becton Dickinson). Cells with polarized mitochondria presented in the top right quadrant of the dot plot due to the formation of JC-1 aggregates, which emit orange color (590 nm) when excited at 488 nm. Cells with depolarized mitochondria emit green color (530 nm) and are visualized in the bottom right quadrant of the dot plot.

Western Blot and Protein Expression Analysis
The protein content of cell extracts was determined by the Bradford assay (Bio-Rad). A total of 20 to 30 μg of protein were electrophoresed on 10% to 15% SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were blocked, incubated with primary antibodies at the appropriate concentration, and subsequently incubated with horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G or goat anti-mouse immunoglobulin G (1:3,000 dilutions; Bio-Rad). Labeled bands were detected with Immun-Star HRP Chemiluminescent Kit, and images were captured. The intensity of the bands was quantitated with the Bio-Rad VersaDoc image system (Bio-Rad). The relative expression of certain protein was determined by dividing the densitometric value of the test protein by that of the control (glyceraldehyde-3-phosphate dehydrogenase or actin).

Immunoprecipitation
Methods used were as previously described with minor modification (38). Briefly, 100 μL of lysates were precleared by incubation with 20 μL of a mixture of protein A and protein G-sepharose packed beads (Santa Cruz Biotechnology) in a rotator at 4°C for 2 h and then with 20 μL of fresh packed beads in a rotator at 4°C overnight. Twenty micrograms of anti–class III β-tubulin polyclonal antibody or control immunoglobulin were then added to the lysate and rotated at 4°C for 2 h. The beads were then pelleted by centrifugation and washed five times with ice-cold lysate buffer before elution of the proteins from the beads in lysate buffer at room temperature for 1 h. The resulted immunoprecipitates were then subjected to SDS-PAGE and Western blot analysis.

siRNA
Melanoma cells were seeded at $1 \times 10^5$ per well in 24-well plates and allowed to reach ~50% confluence on the day of transfection. The PKCδ siRNA and PKCδ siRNA constructs used were obtained as siGENOME SMARTpool reagent (Dharmacon). The nontargeting siRNA control, SiConTRol-Non-targeting SiRNA pool (D-001206-13-20), was also obtained from Dharmacon. Cells were transfected with 50 to 100 nmol/L of siRNA in Opti-MEM medium (Invitrogen) with 5% FCS using Oligofectamine reagent (Invitrogen) according to the manufacturer’s transfection protocol. Forty-eight hours after transfection, the cells were treated with or without docetaxel at 20 nmol/L for the indicated time periods before running apoptotic assays, Bax activation, or mitochondrial membrane potential change measurements. Efficiency of RNA interference was measured by immunoblotting.

Statistical Analysis
Data are expressed as mean ± SE. The statistical significance of intergroup differences in normally distributed continuous variables was determined with Student’s $t$ test. $P > 0.05$ was considered statistically significant.

References


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