Epithelial-to-Mesenchymal Transition and Resistance to Ingenol 3-Angelate, a Novel Protein Kinase C Modulator, in Colon Cancer Cells

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Abstract

Acquired resistance to protein kinase C (PKC) modulators may explain the failure of clinical trials in patients with cancer. Herein, we established a human colon cancer cell line resistant to PEP005, a drug that inhibits PKCα and activates PKCδ. Colo205-R cells, selected by stepwise exposure to PEP005, were >300-fold more resistant to PEP005 than parental Colo205-S cells and were cross-resistant to phorbol 12-myristate 13-acetate, bryostatin, bistatene A, and staurosporine. No PKCα or PKCδ mutation was detected in Colo205-S and Colo205-R cells. Changes in Colo205-R cells were reminiscent of the epithelial-to-mesenchymal transition (EMT) phenotype. Accordingly, Colo205-R cells were more invasive than Colo205-S in Matrigel assays and in mouse xenografts. We also found an increased mRNA expression of several EMT genes, such as those encoding for transforming growth factor-β and vimentin, along with a decreased mRNA expression of genes involved in epithelial differentiation, such as CDH1 (E-cadherin), CLDN4 (claudin 4), S100A4, and MUC1, in Colo205-R compared with Colo205-S cells in vitro and in vivo. Interestingly, high expression of ET-1 was shown in Colo205-R cells and correlated with low sensitivity to PEP005 and staurosporine in a panel of 10 human cancer cell lines. Inhibition of the ET-1 receptor ETR-A with bosentan restored the antiproliferative effects of PEP005 in Colo205-R cells and decreased the invasive properties of this cell line. Exogenous exposure to ET-1 and silencing ET-1 expression using small interfering RNA modulated cell signaling in Colo205-S and Colo205-R. In summary, acquired resistance to PEP005 was associated with expression of EMT markers and activates the ET-1/ETR-A cell signaling. [Cancer Res 2009;69(10):4260–9]

Introduction

Protein kinase Cs (PKC) play a key role in signal transduction (1). PKCs include at least 12 isoforms with varying tissue expression, substrate specificity, and subcellular localization that are related to specialized cellular functions, including cell differentiation, proliferation, apoptosis, motility, adhesion, invasion, and metastasis (2–4). PKCα and PKCδ were shown to play a major role in the balance of proliferation and apoptosis as well as tumor invasion (5). PKCα and PKCδ may be activated by several transmembrane receptors, including G protein–coupled receptors such as endothelin receptors (ETR-A/B) and several tyrosine kinase receptors such as epidermal growth factor receptors (EGFR), platelet derived growth factor receptors, and the tumor necrosis factor α receptor. Following surface receptor stimulation, activation of PKCδ by diacylglycerol may eventually result in apoptosis induction in human cancer cell lines (6, 7). Furthermore, down-regulation of PKCδ activity has been shown to inhibit apoptosis and to induce resistance to DNA-damaging agents (8–10). PKCδ expression was shown to be associated with changes in the invasion capacity of cancer cells (11). Various factors may increase the level and/or activity of PKCδ, including ETR-A and ETR-B activation by ET-1 (12, 13). Phosphorylated PKCα (p-PKCα) may activate the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) 2, Jun NH2-terminal kinase, and nuclear transcription factors nuclear factor-kB (NF-kB) and activator protein-1 (AP-1; refs. 14, 15). Following activation by PKCα, NF-kB, and AP-1 can (a) induce mRNA expression of urokinase-type plasminogen activator and its receptors, (b) induce the secretion of matrix metalloproteinase (MMP) 9 and MMP2, and (c) repress the expression of tissue inhibitor of metalloproteinase (TIMP) 1 and TIMP2, which in turn may increase the invasion capacity of cancer cells. Recently, both PKCα and ET-1 have been shown to play a role in epithelial-to-mesenchymal transition (EMT), leading to an increase of the metastatic potential of cancer cells (16, 17).

PKCs have been considered as attractive targets for the discovery of new anticancer agents. For example, bistratene A, a selective activator of PKCδ, induces apoptosis in human colon cancer cells (18). Consistent with this, PKCδ knockdown by RNA interference (RNAi) and/or dominant-negative mutation was shown to increase proliferation and to inhibit apoptosis in colon cancer cells (18). Similarly, PKCδ inhibition by rottlerin, a drug that competes with ATP, has been shown to prevent the induction of proapoptotic effects of phorbol 12-myristate 13-acetate (PMA) in prostate cancer cells (19, 20). Ingenol derivatives are structurally closely related to...
PMA and have been used in traditional medicines. PEP005 (ingenol 3-angelate), a small molecule extracted from Euphorbia peplus (21), was initially developed as a topical treatment for actinic keratosis or basal cell skin carcinoma and was recently proposed for the treatment of acute myeloid leukemias. PEP005 was shown to inhibit PKCα and to activate PKCδ translocation to cytoplasmic and nuclear membranes in leukemia (21, 22) and in colon cancer models, inducing apoptosis through inhibition of the AKT signaling pathway (23).

Despite evidence of sporadic antitumor activity, resistance to PKC modulators has been a major concern and most drugs investigated in clinical trials had little or no effect on time-to-tumor progression and survival (24). Mechanisms by which cancer cells may become resistant to drugs that target PKC are still poorly understood and may require further investigations to help improve patient selection in clinical trials. Colorectal cancer remains a leading cause of death both through local invasion and distant metastases. In this study, we investigated mechanisms associated with resistance to PEP005 and other PKC modulators in human colon cancer cells, aiming to identify relevant molecular markers of resistance. We showed that resistance to PKC modulators was associated with changes in cellular morphology, invasion, and gene expression, which were reminiscent of a mesenchymal phenotype. Our study may help in understanding the mechanisms associated with acquired resistance to PKC modulators and could serve in identifying resistant tumors in clinical trials.

Materials and Methods

Cell lines. Human cancer cell lines, colon (HT29, HCT116, Colo205, and HCC2998), breast (MCF7 and MDA-MB-435), lung (HOP62 and HOP92), and ovarian (OVCAR3 and IGROV1), were obtained from the National Cancer Institute and American Type Culture Collection. Cells were grown as monolayer in RPMI 1640 supplemented with 10% FCS (Invitrogen), HEPES (pH 7.6), 150 mmol/L NaCl, 1% Triton X-100, 2 mmol/L sodium pyruvate, and glutaraldehyde for molecular biology and immunohistochemical analysis. Mouse experiments were approved by the Animal Housing and Traveling (Paris, France). At baseline, 10 mm3 fragments of Colo205-S and Colo205-R cells was amplified by PCR with these primers and sequenced using fluorescent dyeterminators. Sequences were verified by automated DNA sequencing using a DNA sequencing kit (Perkin-Elmer Applied Biosystems).

Real-time reverse transcription-PCR. The theoretical and practical aspects of real-time quantitative reverse transcription-PCR (RT-PCR) using the ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems) have been described in detail elsewhere (27). Results were expressed as n-fold differences in target gene expression relative to the TBP gene (an endogenous RNA control) and relative to a calibrator (1 × sample), consisting of the cell line sample from our tested series that contained the smallest amount of target gene mRNA.

Immunofluorescence. The cells were grown on coverslips at 37°C overnight. The growth medium was then removed; the cells were washed with PBS, fixed in 4% paraformaldehyde for 10 min, permeabilized with PBS/Triton X-100 (1:1,000), and then washed with PBS. Incubation with primary antibodies was performed at room temperature for 1 h (ET-1, 1:200; ETR-A, 1:400) followed by incubation with the secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. The nuclei were stained with 4′,6-diamidino-2-phenylindole and Hoechst 33342 (Molecular Probes). The images were captured and analyzed with a RT Spot Camera and RT Spot Software (Diagnostic Instruments, Inc.).

ET-1-small interfering RNA. Two different ET-1 RNAi oligonucleotides or scrambled small interfering RNA (siRNA) oligonucleotides (100 nmol/L) were transfected into the cells using the BLOCK-iT transfection kit and Lipofectamine 2000 (Invitrogen). Cells were then incubated for 72 h.

ET-1 ELISA. ET-1 protein in cell culture supernatants and lysates was quantified using ELISA (Immuno-Biological Laboratories). Cells (5 × 106/mL) were seeded in six-well plates and supernatants or lysates of cell cultures were collected after 24 h. The liquid was turned yellow by the addition of stop solution. The absorbance of each well was determined at 450 nm using a microplate reader. The lower limit of detection for ELISA was 0.78 pg/mL. Concentrations of ET-1 were calculated using calibrating curves.

Xenograft models. Female (three to six) nu/nu athymic mice were engrafted in the Animal Facility of the Institute of Hematology, Saint-Louis Hospital (Paris, France). At baseline, 10 mm3 fragments of Colo205-S and Colo205-R tumors were xenografted s.c. under xylazine (10 mg/kg) and ketamine (100 mg/kg) anesthesia. Mice were sacrificed at different times; tumors were weighed and stored in liquid nitrogen, alcohol-formalin-acetic acid, and glutaraldehyde for molecular biology and immunohistochemical analysis. Mouse experiments were approved by the Animal Housing and Experiment Board of the French government.
Results

PEP005-resistant Colo205 cells display cross-resistance to several PKC modulators. The antiproliferative effects of 48-hour exposure to PEP005 were determined in IGROV1 (IC_{50}: 200 ± 42 μmol/L), OVCAR3 (IC_{50}: 200 ± 40 μmol/L), MCF7 (IC_{50}: 180 ± 36 μmol/L), HT29 (IC_{50}: 140 ± 28 μmol/L), HCT116 (IC_{50}: 120 ± 24 μmol/L), HOP62 (IC_{50}: 110 ± 32 μmol/L), HOP92 (IC_{50}: 85 ± 17 μmol/L), HCC2998 (IC_{50}: 30 ± 6 μmol/L), MDA-MB-435 (IC_{50}: 3 ± 0.5 μmol/L), and Colo205-S cells (IC_{50}: 0.01 ± 0.001 μmol/L), Colo205-S being the most sensitive cancer cells.

We established the Colo205-R–resistant cell line from Colo205-S cells using a stepwise exposure to increasing PEP005 concentrations. After 3 months of exposure and 44 passages, cells were able to grow in medium containing 3 μmol/L PEP005. Resistance to PEP005 was shown to be nonreversible after ≥20 passages. Cell doubling time (48 hours) was similar in both cell lines and fluorescence-activated cell sorting analysis showed no significant change in cell cycle distribution between Colo205-R and Colo205-S cells (data not shown). Colo205-R cells (IC_{50} >10 μmol/L) were >300-fold more resistant to PEP005 (Fig. 1A, top) than parental Colo205-S cells. The protein and mRNA levels of PKCα were 10-fold lower in Colo205-R than in Colo205-S cells, with no difference in PKCγ mRNA and protein expressions (Fig. 1A, bottom). No mutation of PKCα and PKCγ was detected in Colo205-S and Colo205-R cells (data not shown). As illustrated in Fig. 1B, Colo205-R cells were cross-resistant to all PKCγ activators, including...
PEP005, PMA, bryostatin, and bistratene A. In addition, some level of cross-resistance between PEP005 and staurosporine was also observed in Colo205-R cells. IC50s of 5-fluorouracil, SN38, and oxaliplatin were similar in Colo205-S and Colo205-R cells (data not shown).

Acquired resistance to PEP005 is associated with morphologic changes and increased invasive capacity reminiscent of EMT. Colo205-S cells displayed round shapes (Fig. 2A, top), clustered together, were easily detached from plastic plates, and underwent some level of anoikis. In contrast, Colo205-R cells did not cluster together and lost the round shape, instead showing scattered elongated cells with lamellipodia/filopodia-like formations (Fig. 2A, bottom); these cells were more adherent to plastic plates and seemed to be more resistant to anoikis than Colo205-S cells. Furthermore, Colo205-R cells were >3-fold more invasive than Colo205-S cells in Matrigel assay (Fig. 2B).

Subsequently, Colo205-S and Colo205-R cells were injected s.c. in nude mice. As shown in Fig. 2C, the growth of Colo205-R cells was significantly higher than that of Colo205-S cells (two-sided \( P < 0.05 \) at days 14, 21, and 27). Liver and lung metastases were infrequent with no significant difference between Colo205-S and Colo205-R xenografts. Microscope analysis showed that tumors induced by Colo205-S cells yielded typical well-differentiated adenocarcinomas with glandular structures and with a well-delineated rim of stromal cells (Fig. 2D, a and b). Conversely, Colo205-R–induced tumors were poorly differentiated adenocarcinomas with elongated cells, no glandular formation, and with cancer cells invading the peripheral rim of the tumor in surrounding tissues, including adipose areas and muscles (Fig. 2D, c and d). These data showing that Colo205-R cells display morphologic changes in cultured cells and tumors, as well as increased invasive capacities in vitro and in vivo, were reminiscent of EMT.

Changes in EMT markers induced by PEP005 in cultured Colo205 cells. We investigated mRNA expression levels of a panel of selected EMT markers in cultured Colo205-S and Colo205-R cells. First, we evaluated changes in the mRNA expression profile of several genes known to play a role in EMT in cultured Colo205-S cells exposed to PEP005. PEP005 exposure in Colo205-S cells was shown to increase mRNA expression of several genes encoding for transforming growth factor-\( \beta \) (TGF-\( \beta \)), transcription factors Snail1, Snail2, and TWIST, and the adhesion molecule claudin 4 (data not shown). Conversely, fibronectin and ET-1 mRNA expression decreased in Colo205-S cells after exposure to PEP005. Subsequently, we compared the mRNA expression profiles of genes involved in cellular differentiation and EMT in Colo205-S and Colo205-R cells (Fig. 3A). mRNA encoding for genes frequently
expressed in differentiated colorectal cancer, such as S100A4, a target of the Wnt/β-catenin pathway, and MUC1, encoding for a mucin, was lower in Colo205-R than in Colo205-S cells, suggesting dedifferentiation. We also found that the mRNA expression of several EMT markers of mesenchymal differentiation was increased in Colo205-R cells, including growth factors (TGF-β/TGFB1 and ET-1), transcription factors (Snail1/SNAIL, Snail2/SLUG, and TWIST1), invasion molecules (N-cadherin/CDH2, vimentin, fibronectin1/FN1, ICAM-1, and MMP9), and various other molecular markers of mesenchymal differentiation (TAGLN, ACTA2, and SPARC). Accordingly, mRNA expression of epithelial markers, such as the adhesion molecules claudin 4 (CLDN4), E-cadherin (CDH1), and connexin 32 (Cx32), was decreased in Colo205-R compared with Colo205-S cells. The enzymatic activity of MMP9 and MMP2 was evaluated by gelatin zymography. MMP2 activity was shown to be increased in Colo205-R compared with Colo205-S cells. Although MMP9 mRNA expression was higher in Colo205-R than Colo205-S cells, the enzymatic activity was similar in both cell lines. Consistent with mRNA expression, the protein level of ET-1 was higher, whereas E-cadherin and claudin 4 protein levels were lower in Colo205-R than Colo205-S cells (data not shown).

**EMT markers in Colo205-S and Colo205-R xenografts.** We further investigated mRNA expression levels of a panel of selected EMT markers in Colo205-S and Colo205-R xenografts. The increased invasiveness of Colo205-R xenografts was associated with increased mRNA expression of several genes involved in EMT and invasion and decreased mRNA expression of genes involved in adhesion. As shown in Fig. 3A, mRNA expression levels of several genes involved in epithelial differentiation and adhesion, such as CDH1 (E-cadherin), CLDN4 (claudin 4), S100A4, MUC1, and CX32 (connexin 32), were decreased, whereas mesenchymal markers, including TGFB1 (TGF-β) and vimentin expression levels, were increased in Colo205-R compared with Colo205-S tumors. In addition, Colo205-R xenografts expressed higher mRNA levels of EMT genes, including TWIST and SLUG (Snail2), as well as other genes involved in invasion, such as ACTA2, HMG2A (HMG proteins), PAI1, and SPARC (osteonectin). Unlike those in cultured cells, ET-1 and SNAIL (Snail1) mRNA levels were similar in Colo205-R and Colo205-S xenografts. Immunoassay of tumors

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**Figure 3.** mRNA expression of genes involved in EMT in cultured Colo205-S and Colo205-R cells and xenografts. Quantitative RT-PCR analysis of genes involved in the EMT, including Snail1/SNAIL, MMP9, ICAM-1, TAGLN, FN1, ET-1, TGF-β/TGFB1, SPARC, N-cadherin/CDH1, S100A4, MUC1, and connexin 32/CX32. A, increased mRNA expression of several EMT genes was observed in Colo205-R relative to Colo205-S cells. B, in mice, Colo205-R xenografts expressed decreased levels of several genes involved in epithelial differentiation (top) and increased levels of several genes involved in mesenchymal transition (bottom) compared with Colo205-S tumors (quantitative RT-PCR analysis). Cancer cells in Colo205-S xenograft (C) expressed higher levels of E-cadherin compared with Colo205-R (D) as measured by immunohistochemistry.
showed that both Colo205-S and Colo205-R xenografts expressed epithelial AE1-3 markers. However, Colo205-S tumor cells expressed high membrane staining for E-cadherin (Fig. 3C), whereas no expression of E-cadherin was detectable in Colo205-R xenografts (Fig. 3D).

**Autocrine secretion and exogenous exposure to ET-1 increase resistance to PEP005 in vitro.** Because ET-1 was recently shown to be an important growth factor that may promote EMT in cancer cells (12, 13, 17, 28), we further focused our work on the mRNA and protein expression levels of ET-1 in Colo205-S exposed to PEP005 and in Colo205-R. As described above, Colo205-S cells exposed to PEP005 had a decreased expression of ET-1. Conversely, Colo205-R cells that display a mesenchymal phenotype had an increased expression of mRNA and protein levels of ET-1. ET-1 is synthesized as a large precursor polypeptide, named pre-pro endothelin-1, which is cleaved into an intermediate peptide, the bigET-1. The bigET-1 is then cleaved by an endothelin-converting enzyme (ECE) to produce the mature ET-1 that binds to two high-affinity serpentine receptor subtypes, ETR-A and ETR-B. In this study, we found that ETR-B was not expressed in Colo205-S or Colo205-R cells (data not shown). We observed a diffuse intracellular localization of ET-1 and a predominant membrane localization of ETR-A in Colo205-S and Colo205-R cells (data not shown). As shown in Fig. 4A, mRNA expressions of ECE-1 and ET-1 were increased in Colo205-R compared with Colo205-S cells, whereas ETR-A expression was similar in both cell lines. Western blot analysis showed that ET-1 and ETR-A proteins were both increased in the Colo205-R cells compared with Colo205-S cells (Fig. 4B). In addition, we observed a 3-fold increase of ET-1 concentrations both in conditioning medium and in cell lysate in Colo205-R compared with Colo205-S cells using the ELISA assay (Fig. 4C). The increased concentration of ET-1 was consistent with an increase of ECE-1 mRNA expression level. To test whether ET-1 itself may play a role in resistance to PEP005, we evaluated the antiproliferative effects of PEP005 in Colo205-S cells with and without pretreatment with exogenous ET-1 at a supraphysiologic high concentration of 100 nmol/L that was previously shown capable of activating PKC signaling. As shown in Fig. 4D, preexposure to ET-1 was associated with decreased sensitivity to PEP005 in Colo205-S cells. Colo205-R cells expressed a higher level of EGFR than Colo205-S cells both at mRNA and protein levels. EGFR was activated only in Colo205-S, this activation being inhibited by a 4-hour exposure to ET-1 (Supplementary Fig. S1).

**ET-1 expression is increased in cancer cells resistant to PKC modulators.** To test whether ET-1 may play a role in resistance in other cancer cells, we further measured the mRNA expression of ET-1, along with that of claudin 4 (CLDN4), PKCa, and PKCy, in our panel of 10 cancer cell lines tested for sensitivity to PEP005 and staurosporine. Attempts were made to correlate the expression of these biomarkers with the antiproliferative activity of PEP005 and staurosporine. We showed no clear correlation between IC50 values of PEP005 (Fig. 5A) or staurosporine (Fig. 5B) and mRNA expression of PKCa, PKCy, and claudin 4/CLDN4. Interestingly, the mRNA expression levels of ET-1 were correlated with resistance to PEP005 ($r^2 = 0.62; P = 0.004$) and staurosporine ($r^2 = 0.60; P = 0.006$), with those cancer cells resistant to PEP005 showing the highest expression of ET-1. Thus, the increased expression of ET-1 in Colo205-R cells, and the high expression of ET-1 in cancer cells resistant to PEP005 and staurosporine, suggested that ET-1 may be an important growth factor for cell survival in the presence of PEP005 and staurosporine.
of PEP005. This also suggested that mRNA expression of ET-1 may be regarded as a potential molecular marker of resistance not only in Colo205-R cells with an acquired resistance to PEP005 but also in unselected cancer cells that display various levels of sensitivity to PEP005 and staurosporine.

Changes in signaling induced by PEP005 in Colo205 cells. PEP005 was shown to act primarily through the activation of p-PKC\(\delta\) and inhibition of p-PKC\(\alpha\), leading to the activation of p-Raf (data not shown), p-p38, and p-ERK1/2 MAPK signaling as well as inhibition of p-AKT and activation of p-GSK3\(\beta\) (Fig. 6A). Exposure of Colo205-R cells to PEP005 showed modest effects on PKC\(\alpha\), PKC\(\delta\), p38, ERK1/2, and AKT phosphorylation levels but a strong activation of GSK3\(\beta\). Exposure of Colo205-S cells to ET-1 yielded a slight increase of p-PKC\(\alpha\), down-expression of p-p38 and p-GSK3\(\beta\),

**Figure 5.** ET-1 expression correlates with resistance to PKC inhibitors. mRNA expression of ET-1, claudin 4, PKC\(\alpha\), and PKC\(\delta\) in 10 solid tumor cell lines was correlated with PEP005 (A) and staurosporine (B) IC\(_{50}\)s. Coefficients of correlation were calculated by linear regression analysis.
and no major changes in p-PKC\(\alpha\) and p-S6K. ET-1 exposure prevented the expected inhibition of p-PKC\(\alpha\), limited the increase of p-PKC\(\gamma\), and modified downstream changes in p38, GSK3\(\beta\), and S6K signaling normally induced by PEP005 and PMA in Colo205-S cells (data not shown).

**Effects of inhibition of the ET1/ETR-A axis in Colo205 cells.**

Bosentan is a competitive antagonist of endothelin receptor that was previously shown inhibiting ET-1–induced ETR-A/B activation at concentration ≥80 μmol/L. As shown in Fig. 6B, inhibition of ETR-A with 100 μmol/L bosentan restored sensitivity to PEP005 in Colo205-R cells. Moreover, Colo205-R cells exposed to bosentan had a 10-fold decreased invasive capacity compared with untreated Colo205-R (Fig. 6C). Colo205-R cells were transfected with ET-1-siRNA (siET-1). Transfection with siET-1 selectively decreased ET-1 mRNA and protein levels as well as the extracellular concentrations of ET-1 in Colo205-R cells (data not shown). No significant change of ECE-1, PKC\(\alpha\), and PKC\(\gamma\) total protein levels was detected. Transfection was unstable and Lipofectamine had intrinsic toxicity that did not allow completion of reliable cytotoxicity and invasion assays with siET-1–transfected Colo205-R cells. siET-1 had no relevant effects on baseline signaling and response to PEP005 and PMA in Colo205-S cells. siET-1 transfection of Colo205-R cells yielded no changes in p-PKC\(\alpha\), p-PKC\(\gamma\), p-ERK1/2, or p-p38 but increased p-GSK3\(\beta\). In siET-1–transfected Colo205-R cells, PEP005 was still capable of decreasing p-PKC\(\alpha\) and increasing both p-GSK3\(\beta\) and p-ERK1/2 (Fig. 6D) with almost no effects on PKC\(\gamma\) and p-p38 (data not shown). Altogether, these data show that the ET-1/ETR-A axis modulates cellular response to PEP005 in Colo205 cells.

**Discussion**

In previous studies, it has been shown that PEP005 inhibits PKC\(\alpha\) and activates PKC\(\gamma\), yielding late G1 cell cycle accumulation.
and induction of apoptosis (22). We previously showed that exposure to PEP005 modulates the phosphoinositide 3-kinase (PI3K)/AKT and ERK1/2 signaling through complex mechanisms in Colo205-S cells (23). Caspase-3 cleavage was described as being associated with PKČ activation, leading to apoptosis in cells treated with PEP005. Previous studies have also shown that some cancer cells may be exquisitely sensitive to PEP005, whereas others required high concentrations barely compatible with clinical uses and may be considered as intrinsically resistant to PEP005. Considering that most previous clinical trials with several PKČ inhibitors failed to show consistent antitumor activity (24), it seemed essential to identify molecular mechanisms of resistance that may serve to select patients with tumors unlikely to benefit from PEP005 therapy. In this study, we first selected Colo205-S among a panel of cancer cells as being the most sensitive cancer cell line. We further selected Colo205-R by stepwise increased PEP005 concentrations. Resistance of Colo205-R to PEP005 was nonreversible in drug-free medium after several passages. Interestingly, Colo205-R cells also display cross-resistance to several other PKČ activators, including PMA, bryostatin, and bistratene A, as well as PKČ inhibitors, such as staurosporine. As shown in this study, mRNA expression and protein levels of the target PKČα were found to be decreased in Colo205-R, whereas no difference for PKČβ was observed. However, whereas acquired resistance was associated with down-expression of PKČα, no correlation between PKČα and PKČβ expression and sensitivity to PEP005 was observed in a larger number of human cancer cell lines. Thus far, large-scale genomic screening has failed to identify PKČα and PKČβ mutations in human colon cancer (29). Although no mutation of PKČβ was reported, potential important sites for PKČ mutations were regions coding for tyrosine phosphorylation sites (30). PKČα-D294G mutants were found in pituitary, breast, and thyroid tumors resulting in altered membrane binding, thereby preventing the transduction of several antitumorigenic signals, and increasing the invasiveness of pituitary tumor cells (31). Because PKČα and PKČβ were the primary targets of PEP005, we searched and found no mutation in exons coding for PKČα and PKČβ in Colo205-S and Colo205-R cells. Our data supported the possibility that other genomic events, beyond target mutations, occurred to counteract the cytotoxicity of PEP005 in Colo205-R cells.

In this study, transcription of TGF-β was activated under exposure to PEP005 along with that of some other transcription factors of EMT. Previous studies have shown that TGF-β–related tumor suppressor activity led to apoptosis by repressing the PI3K/AKT/survivin pathway in colon cancer cells (32). We further studied the role of TGF-β in sensitivity to PEP005. We observed that short-duration exposure of Colo205-S cells to exogenous TGF-β had no effect on the phosphorylations of PKČα, ERK1/2, and p38 but increased p-S6 activity. Exogenous exposure to TGF-β had no effect on the cytotoxicity of PEP005 in Colo205-S cells (Supplementary Fig. S2). In addition to direct effects of PEP005 on PKČs, the increased transcription of TGF-β may participate in the inhibition of the PI3K/AKT pathway and contribute to caspase-3/PKČα–dependent apoptosis induced by PEP005 in Colo205-S cells. Continuous exposure to PEP005 induced strong and stable TGF-β overexpression associated with the activation of the transcription machinery, leading to the overexpression of several transcription factors, such as Snail1, Snail2, and TWIST, as well as cellular changes that were reminiscent of EMT in Colo205-R. The tumor promoter function of TGF-β has been associated with its ability to induce EMT, which confers resistance to the apoptotic effects of TGF-β. Thus, our data strongly suggest that Colo205-R cells have escaped from both PKČα-dependent and TGF-β–mediated mechanisms that initially participated in apoptosis induced by PEP005 and a broad number of PKČ modulators in Colo205-S cells.

EMT plays a central role in cancer progression, allowing cells to develop invasive competences by changing adhesive properties, by activating proteolysis and motility (33), and by repressing the expression and/or the function of intercellular adhesion molecules, including E-cadherin, claudin 4, and connexin 32 (34–37). Consistently, Colo205-R cells were poorly differentiated and had an increased invasive potential in vitro and in vivo. We also showed that resistance to PKČ modulators was associated with changes in genes involved in EMT, including the overexpression of vimentin, and genes involved in invasion (N-cadherin, ICAM-1, MMP2, MMP9, and ACTA2), along with decreased mRNA expression of several genes involved in adhesion of epithelial cells (E-cadherin, CLDN4, and CX32). ET-1 is a small vasoactive peptide that was shown to be expressed at high levels in plasma and tumor tissue samples from patients with colorectal cancer (38). ET-1 stimulates proliferation of colorectal cancer cells via the ETR-A receptor and subsequent activation of pertussis toxin–sensitive Go/Gi proteins, PI3K, PKČ, and transactivation of the EGFR. ET-1 was also previously shown to play an important role in EMT (12, 17, 39). In this study, we found that ET-1 and ETR-A were expressed in Colo205-S and Colo205-R cells. However, the ET-1 level was higher in Colo205-R cells, which display the EMT phenotype, than in Colo205-S. In addition, pharmacologic inhibition of ETR-A with bosentan reduced the invasive capacity of Colo205-R cells. This finding fits with previous data by Rosano and colleagues (12, 17) showing that ET-1 was a key factor in promoting EMT in ovarian human cancer (39). These data also seemed to be consistent with previously published results showing reciprocal activation of ET-1 and TGF-β–tumor promoter function in cells undergoing EMT (28). Data previously published have shown that ET-1/ETR-A promotes nuclear localization and transcription of Snail, as well as a Snail and β-catenin protein stabilization, thereby activating a transcriptional program that regulates EMT determinants. Snail1 represses E-cadherin expression by binding the E-boxes present in its promoter in vitro and in vivo (39, 40). Our data also showed that Colo205-R cells growing in xenografts lost the overexpression of ET-1, suggesting that ET-1 overexpression may only be transiently required during the dedifferentiation process that promotes local invasion and may not be required anymore in established tumors. ET-1 has been previously shown to play a role in preventing apoptosis in several tumor models (12). In this study, we showed that Colo205-S cells expressed ETR-A and responded to ETR-A stimulation by exogenous ET-1 by reducing phosphorylation of p38 and GSK3β. Exogenous ET-1 was also shown to reduce the antiproliferative effects of PEP005 in Colo205-S. Our data suggested that baseline ET-1 may not be sufficient to counteract apoptosis induced by PEP005 in Colo205-S cells but that exogenous activation of ETR-A by ET-1 may trigger changes in cell signaling that counteract modulations of PKČ by PEP005. Colo205-R cells that become resistant to PEP005 also activate transcription of ET-1, a mechanism that induces the autocrine stimulation of cells through ET1/ETR-A signaling and that will in turn help activation of the EMT machinery. We have shown that Colo205-R, as well as several other cancer cells resistant to PEP005, displayed a high level of ET-1 and that ET-1 expression may be regarded as a molecular marker of resistance to several PKČ inhibitors. Furthermore, switching off the ET-1/ETR-A survival signaling pathway, using pharmacologic
inhibition of ETR-A, restored the sensitivity to PEP005 in Colo205-R cells. From these data, it is likely that autocrine activation of ET-1/ETR-A may counteract the apoptotic effects of PKC modulators in Colo205-R cells by switching on survival mechanisms that take over when PKCα signaling is down-regulated. Indeed, the blockage of ET-1/ETR-A-dependent signaling using receptor antagonists may also be considered as an interesting approach to restore sensitivity in cells resistant to PKC modulators.

In summary, our work shows that cancer cells resistant to PEP005 may develop cross-resistance to several other PKC modulators. Acquired resistance to PEP005 was associated with EMT. These molecular changes could not only serve as molecular markers of resistance but also seem to be associated with an increased invasive capacity of cancer cells in vitro and in vivo. Our study may help in understanding the mechanisms associated with resistance to PKC modulators and could serve in identifying resistant tumors in clinical trials. Colo205-R cells also represent a potentially useful tool in gaining insights into the mode of action of PEP005 in tumor cells and selecting non-cross-resistant PEP005 structural analogues, as well as in investigating and developing methods to prevent resistance to this drug or other targeted therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 7/28/08; revised 3/10/09; accepted 3/10/09; published OnlineFirst 5/5/09.

Grant support: Association d’Aide à la Recherche et l’Enseignement en Cancérologie (Clichy, France). A. Ghoul was a grant recipient for a joint support from ministries of research and industry (Conventions Industrielles de Formation par la Recherche).

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We thank Dr. Shahin Emami (Institut National de la Sante et de la Recherche Medicale U673) for her critical reading of the manuscript and Dominique Vidaud, Keyvan Rezaei, Nasser Assaf, and Christophe Le Tourneau for technical assistance.

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