Sphingosine Kinase-1 as a Chemotherapy Sensor in Prostate Adenocarcinoma Cell and Mouse Models

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Abstract

Systemic chemotherapy was considered of modest efficacy in prostate cancer until the recent introduction of taxanes. We took advantage of the known differential effect of camptothecin and docetaxel on human PC-3 and LNCaP prostate cancer cells to determine their effect on sphingosine kinase-1 (SphK1) activity and subsequent ceramide/sphingosine 1-phosphate (S1P) balance in relation with cell survival. In vitro, docetaxel and camptothecin induced strong inhibition of SphK1 and elevation of the ceramide/S1P ratio only in cell lines sensitive to these drugs. SphK1 overexpression in both cell lines impaired the efficacy of chemotherapy by decreasing the ceramide/S1P ratio. Alternatively, silencing SphK1 by RNA interference or pharmacologic inhibition induced apoptosis coupled with ceramide elevation and loss of S1P. The differential effect of both chemotherapeutics was confirmed in an orthotopic PC-3/green fluorescent protein model established in nude mice. Docetaxel induced a stronger SphK1 inhibition and ceramide/S1P ratio elevation than camptothecin. This was accompanied by a smaller tumor volume and the reduced occurrence and number of metastases. SphK1-overexpressing PC-3 cells implanted in animals developed remarkably larger tumors and resistance to docetaxel treatment. These results provide the first in vivo demonstration of SphK1 as a sensor of chemotherapy. (Cancer Res 2005; 65(24): 11667-75)

Introduction

Sphingolipids represent a potent class of apoptosis regulators in cancer cells (1–3). Ceramide mediates apoptosis in response to a wide array of anticancer treatments (1, 4). In contrast, sphingosine 1-phosphate (S1P), synthesized by sphingosine phosphorylation, antagonizes the stresses that induce ceramide generation (5). The opposing directions of ceramide- and S1P-mediated signaling gave birth to the concept of a ceramide/S1P biostat and the postulate that the ratio between these two lipids could determine the cell fate (5). The levels of ceramide and S1P can be regulated by sphingosine kinase-1 (SphK1), which converts proapoptotic ceramide and sphingosine into prosurvival S1P (2). SphK1 is an oncogenic enzyme (6) whose overexpression can abrogate apoptosis (7–10). Furthermore, the content of SphK1 mRNA was found significantly higher in various tumor tissues than in the healthy counterparts (11). Finally, consistent with the idea that ceramide is a mediator of apoptosis, deficiency in ceramide generation has been linked to tumor cell resistance to cancer therapy (12–16). Interestingly, a relationship has been established in prostate cancer cell culture models between SphK1 and resistance to irradiation. It was shown that SphK1 activity was not affected by ionizing radiations in radioresistant LNCaP cells, whereas SphK1 was markedly inhibited in radiosensitive TSU cells (15). These results suggested that SphK1 inhibition was associated with radiation-induced apoptosis in sensitive cells to halt production of S1P, the antiapoptotic sphingolipid.

To further substantiate a role for SphK1 as an indicator of tumor cell sensitivity or resistance, we have now examined its involvement in susceptibility to antineoplastic agents in prostate cancer cell lines and mouse models.

Prostate cancer is the leading cancer in the male population, targeting one third of overall cancer patients. The management of prostate cancer remains complex. A diverse array of treatment options, including surgery, hormonotherapy, radiation therapy, and chemotherapy, is available. Advances in the treatment of late-stage prostate cancer have been limited in the past by the lack of effective agents. Estramustine-based regimens, camptothecins, and doxorubicin-based combinations have mainly been palliative in nature (17, 18). Docetaxel has recently been introduced for the treatment of metastatic prostate cancer, following demonstration of its survival benefit in advanced hormono-resistant prostate cancer patients (19, 20).

The participation of sphingolipid metabolism in taxane-mediated apoptosis has been suggested. Taxol (paclitaxel) has been shown to induce ceramide generation in neuroblastoma (21), breast cancer cells (22, 23), renal carcinoma (24), and prostate cancer cells (25). Interestingly, S1P could block Taxol-induced apoptosis in ovarian cancer cells (26). We, therefore, investigated the ability of SphK1 to regulate apoptosis in LNCaP and PC-3 prostate cancer models in response to docetaxel (Taxotere), in comparison with camptothecin, a well-known inducer of apoptosis in LNCaP cells with little clinical relevance (27–30); however, similarly to taxanes, camptothecin induces ceramide generation during apoptosis in several cancer models (30–34).

PC-3 and LNCaP cells exhibit different sensitivities to camptothecin and taxanes. Indeed, LNCaP cells seem to be quite sensitive to 0.1 to 1 μmol/L doses of camptothecin (27–30, 35), whereas the reported sensitivity of PC-3 cells to camptothecin ranges from moderately sensitive (35–38) to completely resistant (28). Interestingly, the latter authors linked PC-3 resistance to the absence of ceramide generation and resensitized these cells to apoptosis by extracellular ceramide addition (28). They also reported that camptothecin-induced apoptosis in LNCaP cells was mediated by ceramide (28). In contrast, docetaxel was found to trigger

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apoptosis in both LNCaP and PC-3 prostate cancer cells, although Wang and Wieder (39) clearly showed that PC-3 cells were much more sensitive to nanomolar doses of docetaxel than LNCaP cells. Of note, PC-3 and LNCaP also differ in hormonal requirements as LNCaP growth is exclusively dependent on androgens, whereas PC-3, derived from a patient who died from hormono-resistant prostate cancer, is not. In this respect, the two cell lines exhibit contrasting sensitivities to hormone therapy and chemotherapy, mimicking the progression from hormono-sensitive to hormone refractory disease that is observed in human medicine.

We took advantage of the known differential effect of camptothecin and docetaxel on human PC-3 and LNCaP prostate cancer cells to determine the relevance of SphK1 activity and the ceramide/SIP ratio, in relation with cell survival after chemotherapy in vitro and in vivo. We report that docetaxel and camptothecin treatment of PC-3 and LNCaP cells induced inhibition of SphK1 and elevation of the ceramide/SIP ratio solely in cells sensitive to these drugs. In addition, the inhibition of SphK1 mimicked the effects of docetaxel and camptothecin, whereas its overexpression rendered sensitive cells resistant to chemotherapy. Furthermore, these findings could be confirmed in vivo using an orthotopically transplanted fluorescent PC-3 model in mouse. This experimental animal model enabled us to show the contribution of the ceramide/SIP balance in anticancer therapy, for the first time in vivo, thereby supporting the notion that SphK1 inhibition could be a central effector mediating chemotherapy-induced apoptosis in prostate cancer.

Materials and Methods

Cell lines. Human prostate cancer PC-3 and LNCaP were cultured in RPMI 1640 containing 10% fetal bovine serum. FLAG epitope–tagged wild-type human SphK1 cDNA (40) was used for stable transfection by Effectene reagent (Qiagen, Courtabœuf, France). Mass pools of stable transfectants were selected with 1 mg/mL G418. Empty vector– and wild-type human SphK1–transfected cells were designated as PC-3/neo, LNCaP/neo, PC-3/SphK1, and LNCaP/SphK1, respectively. Experiments were conducted in the absence of selection. Stably transfected green fluorescent protein (GFP)-tagged PC-3 cells used for in vivo experiments were maintained in medium containing 1 mg/mL G418.

Materials. Medium, serum, and antibiotics were obtained fromInvitrogen. Docetaxel (Taxotere, Cergy-Pontoise, France) and silica gel TLC (Partisil LK6D) plates were from Whatman International (Maidstone, United Kingdom).

Cell viability, flow cytometry, and staining of apoptotic nuclei. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay as previously described (41). Flow cytometric analysis was done on cells double-stained with Annexin V–FITC and propidium iodide using a FACSCalibur flow cytometer (Becton Dickinson, Le-Pont-de-Claix, France). Apoptosis was visually assessed by double staining cells with Syto 13 (1 μmol/L) and propidium iodide (6 μg/mL) for 5 minutes at 37°C. The cells were then examined with a Leica fluorescent microscope and apoptotic cells were distinguished by condensed, fragmented nuclear regions.

Western blot analysis. Western blotting was done as previously reported (42). Mouse anti-FLAG (Sigma) and mouse anti-i-actin (Sigma) were used as primary antibodies. Proteins were visualized by enhanced chemiluminescence detection system (Pierce, Brabière, France) using anti-mouse horseradish peroxidase–conjugated IgG (Bio-Rad, Marne La Coquette, France).

Sphingosine kinase-1 assay and mass measurements of sphingolipids. SphK1 activity was measured according to the procedure of Liu et al. (43) under conditions optimal for SphK1 activity. Ceramide and SIP levels were measured and normalized to total cellular sphingolipids as previously described (44).

RNA interference. Transient interference was achieved by double-stranded SphK1-specific small interfering RNA (siRNA) 5′-GGOCAAGGG-CUUGGCCAGCUdT(TT)−3′ and 5′-GAGCUAGACCUUGCCCGCAT(TT)−3′ or scrambled siRNA 5′-UUCUGCGAACUGUGACCUdT(TT)−3′ and 5′-ACGU-GACACUGUCGAGAAA(TT) (Qiagen) using OligofectAMINE reagent (Invitrogen).

Animals. NMRI/Nu (nu/nu) 6-week-old male mice were obtained from Janvier (Le Genest-St-Ise, France). At 7 to 8 weeks of age, the animals were used in accordance with the principles and procedures outlined in Council Directive 86/609/EEC. All animal studies were approved by the local Animal Care and Use Committee.

Surgical orthotopic implantation of PC-3 cells. Intraprostatic human prostate cancer xenografts were established in nude mice by surgical orthotopic implantation (45). Mice were anesthetized by isoflurane inhalation and placed in the supine position. A lower midline abdominal incision was made and 20 μL tumor cell suspension (1 × 106 cells) was injected into the dorsal lobe of the prostate using a 30-gauge needle and glass syringe. The surgical wound was closed in two layers with 4-0 Dexon interrupted sutures. All procedures were done with a dissecting microscope. Three weeks after surgical orthotopic implantation, the mice were randomized into different groups for three treatments with irinotecan or docetaxel. One group served as the negative control and received a sham injection instead of an active treatment.

Autopsy, histology and, in vivo fluorescence imaging. Two days after the final treatment, all mice were anesthetized, percutaneous GFP imaging analysis was done, and then mice were euthanized with carbon dioxide asphyxiation for direct internal imaging. A lower midline incision was made to access the abdominal and the thoracic cavities. The fluorescent primary tumor was removed en bloc with the seminal vesicles and a meticulous fluorescence-assisted exploration was conducted to establish the presence of periarterial nodal extensions, as well as adrenal, liver, and lung metastases. The primary tumors were then removed, saved, and routinely processed for H&E histology to confirm the nature of the disease or processed for sphingolipid analyses.

GFP fluorescence was detected with a Leica MZFL III fluorescence stereomicroscope (Leica Microsystems, Wetzlar, Germany). High-resolution 16-bit images (1,392 × 1,040 pixels) were captured by a thermoelectrically cooled charge-coupled device camera (CoolSNAP HQ, Roper Scientific, Evry, France). To visualize the whole tumor and lymph nodes or micrometastases, fluorescence-assisted exploration was conducted to establish the presence of periaortic nodal extensions, as well as adrenal, liver, and lung metastases. The primary tumors were then removed, saved, and routinely processed for H&E histology to confirm the nature of the disease or processed for sphingolipid analyses.

Statistical analysis. The statistical significance of differences between the means of two groups was evaluated by unpaired Student's t test. The frequencies of metastases between two groups were compared using Fisher's exact test. Differences in the number of metastases per mouse were examined using a nonparametric Wilcoxon rank sum test. All statistical tests were two-sided and the level of significance was set at P < 0.05. Calculations were done using Instat (GraphPad Software, San Diego, CA).

Results

PC-3 and LNCaP exhibit different sensitivities to docetaxel and camptothecin. In line with previous studies (27–30, 35), a 72-hour camptothecin treatment induced 60% loss of cell viability in
LNCaP cells (Fig. 1A). In contrast, the 20 nmol/L docetaxel treatment was 60% less efficient consistent with Wang's observation that docetaxel was a weak inducer of apoptosis in LNCaP even at doses up to 1 μmol/L (39). As depicted in Fig. 1A, a 50% loss of viability was reached after 120 hours of treatment with docetaxel and after <48 hours with camptothecin. However, these two chemotherapeutics had very different effects on PC-3 cells, with docetaxel inducing a profound decrease in cell viability and camptothecin having less effect (Fig. 1A). In contrast to Wang et al. (28), but in agreement with others (27, 29, 30, 35, 38), we did not find PC-3 cells totally resistant to camptothecin treatment. Flow cytometry analysis revealed that the decrease of cell viability observed in both cell lines could be attributed to apoptosis (Fig. 1B) as confirmed by Syto13/propidium iodide staining (Fig. 1C).

**Apoptosis is correlated with sphingosine kinase-1 inhibition and with a shift of intracellular lipid balance toward ceramide production.** The camptothecin treatment of “camptothecin-sensitive” LNCaP cells resulted in a profound time-dependent SphK1 inhibition (Fig. 1D). In contrast, no significant SphK1 inhibition was induced in the relatively resistant PC-3 cells even after 48 hours of camptothecin treatment. Prolonged camptothecin treatment could, however, induce a limited inhibition of SphK1 (Fig. 1D), which was enough to trigger a subsequent loss of cell viability (Fig. 1A). Mirroring camptothecin-treated PC-3 cells, the treatment of LNCaP cells with docetaxel exhibited small SphK1 activation (Fig. 1D). After 72 hours, SphK1 activity was, however, decreased by 25% (Fig. 1D), resulting in apoptosis at later times (Fig. 1A). In the docetaxel-sensitive PC-3 cells, treatment induced SphK1 inhibition as early as 6 hours and a 72-hour treatment decreased SphK1 activity by 70% (Fig. 1D), with a simultaneous 60% decrease in cell viability (Fig. 1A). Docetaxel and camptothecin displayed no SphK1 direct inhibiting properties as neither drug was able to inhibit the SphK1 activity in test tube (data not shown).

We next measured the ceramide and S1P contents after docetaxel and camptothecin treatment. Chemotherapy-induced SphK1 inhibition resulted in significant changes in the ceramide and S1P levels of both cell lines. In PC-3 cells, a 24-hour treatment with docetaxel resulted in 30% SphK1 inhibition (Fig. 1D), which was paralleled by a 25% increase in ceramide content (Fig. 1F) and a 35% decrease in S1P (Fig. 1E). This pattern was amplified overtime and, after 72 hours of treatment, a 6-fold increase in the ceramide/S1P ratio in PC-3 treated with docetaxel was observed compared with a <2-fold

![Figure 1. Docetaxel and camptothecin effects on cell viability, SphK1 activity, and intracellular lipid balance in LNCaP and PC-3 cells. LNCaP and PC-3 cells were incubated in the presence of 500 nmol/L camptothecin or 20 nmol/L docetaxel for the indicated times. A, cell viability was assessed by MTT test. Points, mean of eight independent experiments; bars, SD. The two-tailed P value between the means of docetaxel- and camptothecin-treated cells is <0.001. B, Annexin V-propidium iodide staining of PC-3 cells incubated for 48 hours with camptothecin or docetaxel. C, representative images of 48-hour docetaxel- and camptothecin-treated cells stained with Syto13-propidium iodide. D to F, cells were incubated with 500 nmol/L camptothecin (black columns) or 20 nmol/L docetaxel (white columns) for the indicated times, then tested for SphK1 activity (D), S1P (E), and ceramide (Cer, F) levels. Columns, mean of five independent experiments done in quadruplicate; bars, SD. P < 0.001 unless otherwise indicated. **, P < 0.01; *, P < 0.1; ns, not significant. Basal SphK1 activities were 11.3 ± 1.7 and 42.7 ± 5.5 pmol/mg/min for LNCaP and PC-3, respectively.](https://www.aacrjournals.org/doi/abs/10.1158/0008-5472.CAN-05-0482)

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increase with camptothecin (calculated from the relative amounts of ceramide and S1P levels shown in Fig. 1E and F, respectively; 1.77:0.28 versus 1.35:0.75). This dramatic difference clearly reflects the differential responsiveness of PC-3 to the two drugs. Similarly, the initial SphK1 activation by camptothecin in PC-3 or by docetaxel in LNCaP cells was well correlated with a proportional increase in S1P and decrease in ceramide, which was reversed after the inhibition of SphK1 activity occurring after longer incubation times (Fig. 1). In the LNCaP cells, camptothecin-induced cell death was paralleled by an 8-fold increase of the ceramide/S1P ratio (3.45:0.43) by 72 hours (Fig. 1). In contrast, the increase induced by docetaxel was <3-fold (2.11:0.70) and confirmed its weaker effect on LNCaP cell viability (Fig. 1). Thus, a reverse correlation during chemotherapy between SphK1 activity and the extent of cell death seems to exist during chemotherapy, which is apparent as a dramatic change in the ceramide/S1P ratio.

Sphingosine kinase-1 overexpression shifts the lipid biostat toward prosurvival sphingosine 1-phosphate and renders PC-3 and LNCaP cells less sensitive to chemotherapy. Because an inhibition of SphK1 is observed during chemotherapy-induced apoptosis, transfection of PC-3 and LNCaP cells with this enzyme might render these cells resistant to anticancer treatment. Transfection efficiency was verified by Western blotting with FLAG antibody (Fig. 2A). The SphK1 activity of both SphK1-overexpressing LNCaP (Fig. 2B) and PC-3 (Fig. 2C) was increased to ~800 pmol/mg protein/min (i.e., ~20- and 75-fold higher, respectively, compared with that of empty vector-transfected cells). This increase of SphK1 activity led to a shift in the sphingolipid balance and notably in the ceramide/S1P ratio. As shown in Fig. 2B and C, the overexpression of SphK1 in both PC-3 and LNCaP cell lines diminished the level of total intracellular ceramide in resting cells by 25% and 40%, respectively. The basal S1P level was increased by 75% and 50%, respectively.

The role of SphK1 inhibition in apoptosis induction was confirmed by cell viability assays, which showed that SphK1-overexpressing LNCaP cells were 75% more resistant to camptothecin than LNCaP cells transfected with empty vector (Fig. 2D). LNCaP sensitivity to docetaxel was only slightly altered by SphK1 overexpression, which correlates well with the lower degree of SphK1 inhibition induced by this drug (Fig. 1D). Similarly, SphK1 overexpression in PC-3 cells only induced slight resistance to camptothecin (which has little effect on SphK1 activity; Fig. 1D), whereas PC-3/SphK1 cells were almost 2-fold more resistant to the sphingosine kinase–inhibiting docetaxel (Fig. 2E).
To verify the mode of action of SphK1 overexpression on cell protection, we then studied the SphK1 inhibition induced by both chemotherapies in Neo- and SphK1-overexpressing cells. Camptothecin-induced SphK1 inhibition was lower in LNCaP/SphK1 than in LNCaP/Neo cells (Fig. 3A), which translated into a lower S1P decrease (Fig. 3B) and lower ceramide increase (Fig. 3C). This was illustrated by a time-dependent decrease of the ceramide/S1P ratio (from an almost 8-fold increase to 3.5-fold at 72-hour time point, 3.45:0.46 versus 2.59:0.70, respectively). Similarly, PC-3/SphK1 showed significant resistance to docetaxel treatment (Fig. 2E), which was consistent with SphK1 inhibition (Fig. 3D), S1P diminution (Fig. 3E), and ceramide elevation (Fig. 3F). As a result, the ceramide/S1P ratio in PC-3/SphK1 was >3-fold lower than in PC-3/neo cells treated with docetaxel for 72 hours (1.98:0.20 versus 1.67:0.58, respectively).

Manipulating the ceramide/sphingosine 1-phosphate ratio by sphingosine kinase pharmacologic inhibitor or RNA interference promotes apoptosis. To establish proof of concept that SphK1 down-regulation has a critical effect on the cytotoxicity of chemotherapeutics, we first examined the effects of siRNA targeted against SphK1 (10, 46) on SphK1 activity and PC-3 cell viability. SphK1 activity was decreased by >80% compared with scrambled siRNA (Fig. 4A). This was further illustrated by decrease in S1P content (Fig. 4A, inset). Western blot analysis revealed a substantial down-regulation of the SphK1 protein in PC-3/SphK1 cells after siRNA treatment for 72 hours (Fig. 4A). The decrease in SphK1 activity was accompanied by a significant loss of cell viability that could last for at least 120 hours posttransfection with hSphK1 siRNA (Fig. 4B). These data clearly imply that SphK1 is required for cell survival and that the reduction of SphK1 may be crucial to the execution of apoptosis as recently reported in breast adenocarcinoma (46) and leukemia cells (47). The role of the ceramide/S1P balance was further examined by performing experiments with 2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole, also known as compound II, which is currently the most selective SphK1 pharmacologic inhibitor available (11). Figure 4C and E, respectively, shows that compound II induced a dramatic decrease in SphK1 activity in both LNCaP and PC-3 cells that culminated after 72 hours. As anticipated, SphK1 inhibition was correlated with an induction of apoptosis (Fig. 4D and F). Conversely, both LNCaP/SphK1 and PC-3/SphK1 cells exhibited less SphK1 inhibition after treatment with compound II (Fig. 4C and E), which was coupled with a much lower ceramide/S1P ratio (data not shown). Accordingly, both SphK1-overexpressing prostate cancer cell lines showed robust resistance toward compound II–induced cell death (Fig. 4D and F).

In vivo efficacy of chemotherapeutics in human prostate cancer cells is linked to the ceramide/sphingosine 1-phosphate balance. The role of SphK1 and the ceramide/S1P biostat in response to chemotherapeutics was then analyzed in vivo using the surgical orthotopic implantation of human PC-3 cells overexpressing GFP (48). The fluorescence of orthotopic tumors was strong enough to be observed externally (Fig. 5A, left). A whole-body open image of a representative sham-treated animal revealed the bright fluorescence of both primary tumor and the periaortic lymph node.
node metastases, indicating a disseminating disease (Fig. 5A, right). Higher-magnification microscopy is shown in Fig. 5B. Metastatic sites included the periaortic lymph nodes (Fig. 5B, short arrows), adrenals (Fig. 5C), liver (Fig. 5D), and lungs (Fig. 5E). The histology of the primary tumor was consistent with poorly differentiated prostate cancer (Fig. 5F). Significant smaller tumors were seen in animals treated for 2 weeks with 70 mg/kg irinotecan or 20 mg/kg docetaxel (Fig. 5F). The tumor volume obtained by measured visualized fluorescence (Fig. 5G) may serve as a reliable surrogate for tumor mass measurement (Fig. 5H). Indeed, both methods showed that docetaxel was much more effective than irinotecan. Moreover, the effect on primary tumor growth was paralleled by a significant limitation of metastasis occurrence in the docetaxel-treated group (66%) compared with 100% in both the sham- and irinotecan-treated groups (Table 1).

Having established the superior efficacy of docetaxel in PC-3 cells both in vitro and in animal studies, we next evaluated the SphK1 activity and sphingolipid content of primary tumor samples obtained from treated mice. We observed a 30% (P = 0.0329) and a 50% (P = 0.0007) decrease in SphK1 activity in irinotecan- and docetaxel-treated animals, respectively (Fig. 5J). A dramatic reduction in the SIP content (Fig. 5J) was observed in both irinotecan- and docetaxel-treated animals (30%, P = 0.0256 and over 40%, P = 0.0044; respectively) compared with sham-treated mice. As shown in Fig. 5K, the ceramide content was dramatically increased in docetaxel-treated mice compared with untreated (40% increase, P = 0.0080) or irinotecan-treated animals (P = 0.0329). In consequence, the ceramide/SIP ratio in docetaxel-treated animals was 2.4-fold higher than in sham-treated mice, compared with a 1.6-fold higher with the irinotecan treatment. These in vivo data therefore support the hypothesis suggested in vitro (Fig. 1) that drug efficacy is indeed correlated with the ceramide/SIP ratio status.

**Sphingosine kinase-1 overexpression in PC-3 cells promotes tumorogenesis in nude mice and impedes docetaxel effects on tumor.** As SphK1 enhanced survival in PC-3 cells in response to docetaxel in vitro, it was of interest to determine its effect in an animal model. First, we found that PC-3/SphK1 tumors were markedly larger than those of PC-3/Neo cells (Fig. 6A). Indeed, 5 weeks after orthotopic implantation, mice injected with PC-3/SphK1 cells exhibited a 2.5-fold increase in tumor size (n = 6; P = 0.0003). Second, the overexpression of SphK1 rendered tumors less sensitive to docetaxel treatment. Indeed, tumor size shrank by almost 70% in PC-3/Neo implanted tumors compared with ~35% increase in PC-3/SphK1 cells (Fig. 6A). As shown in Fig. 6B, SphK1 activity was strongly inhibited by docetaxel treatment of PC-3/Neo orthotopic tumors. This considerable inhibition was not observed
in PC-3/SphK1 tumor samples (Fig. 6B). Accordingly, the ceramide elevation triggered by docetaxel treatment was attenuated in animals implanted with PC-3/SphK1 (Fig. 6C). Because of this, the increase in ceramide/S1P ratio was only 1.6-fold in PC-3/SphK1 tumors compared with >3-fold in PC-3/neo tumors as already observed in vitro (Fig. 3E and F).

**Conclusion**

These results show that SphK1 activity, and its effects on the ceramide/S1P sphingolipid balance, can be regarded as a "sensor" of chemotherapies in prostate cancer, reflecting drug efficacy both in vitro and in vivo. Our study also helps to clarify the findings of numerous reports that LNCaP and PC-3, two widely used prostate cancer cell lines, respond differently to camptothecins and taxanes.

SphK1, like many prosurvival proteins, is down-regulated upon apoptotic stimulus. We have already reported that apoptosis induced by the irradiation of TSUPr1 prostate cancer cells occurred concurrently with SphK1 activity inhibition, a situation that was not seen in radioresistant LNCaP cells (15). In this study, we establish that the intensity of SphK1 inhibition can predict the outcome of prostate cancer chemotherapy. A low degree of SphK1 inhibition resulted in poorer drug efficacy, whereas a robust SphK1 inhibition was followed by a strong antitumoral effect. The extent of SphK1 inhibition was reflected by the status of the ceramide/S1P ratio. We actually found a 2- to 3-fold increase in the ceramide/S1P ratio of the least chemosensitive cells in contrast to a 6- to 8-fold surge in those that were most chemosensitive.

Camptothecins are believed to target topoisomerase-1, of which the mRNA is increased in tumor samples (49). However, clinical tests using various camptothecin derivatives showed limited efficacy in the treatment of hormono-resistant prostate cancer patients. Cell culture–based experiments showed that camptothecin was more active on non–hormono-resistant prostate cancer cells, such as the androgen-sensitive LNCaP, than on hormono-resistant prostate cancer models, such as PC-3 (28, 35, 38). Owing to the fact that LNCaP are p53 wild-type cells, it was proposed that camptothecin needed a functional p53 to function as p53 overexpression in PC-3.

**Figure 5.** Effects of docetaxel and irinotecan on growth of established GFP-expressing human PC-3 prostate cancer tumors in nude mice. A, percutaneous (left) and open whole-body images (right) show a single, typical, control mouse at autopsy 5 weeks after surgical orthotopic implantation of PC-3/GFP cells. A representative primary tumor and periaortic lymph node (B), periaeral (C), liver (D), and lung (E) metastases imaged at day 37 as described in Materials and Methods. Long arrow, primary tumor; short arrows, metastases. Three weeks after surgical orthotopic implantation, mice were randomized into three groups of six to eight mice each. These mice were then subjected to three weekly 20 mg/kg docetaxel, 70 mg/kg irinotecan treatments, or a sham treatment by i.p. injections. F, excised tumor from sham-treated animals visualized by H&E staining. Original magnification, ×200. G, representative fluorescent primary prostate tumors from sham-, irinotecan-, and docetaxel-treated groups at the time of autopsy. Red arrows, primary orthotopic GFP tumor; yellow, bladder. G, tumor volume calculated as described in Materials and Methods. H, tumor mass of excised primary tumor. Columns, means from 12 to 13 animals; bars, SE (G and H). SphK1 activity (I), S1P (J), and ceramide (K) levels were measured in tissue extracts obtained from sham-, irinotecan-, and docetaxel-treated animals. Columns, mean from 12 to 13 animals; bars, SE.
Second, the pharmacologic inhibition of SphK1 was able to kill both androgeno-sensitive (LNCaP) and hormono-resistant prostate cancer cells (PC-3), notably by tilting the ceramide/S1P biostat toward ceramide. This clearly shows that the specific inhibition of SphK1 can lead to the death of prostate cancer cells regardless of their initial sensitivity/resistance status to a given drug or p53 status.

Our studies show that in vitro findings can be translated in vivo by using an orthotopic fluorescent PC-3 model established in nude mice. Orthotopic models of prostate cancer involve the development of human cancer cell suspensions injected into the organ. These xenografts are thus growing in their native milieu and may lead to locoregional growth and spontaneous distant metastasis. They are, therefore, more accurate as cancer models than s.c. xenografts, which typically do not metastasize. Furthermore, GFP labeling markedly improved visualization of the tumor and metastases and ensured a very high resolution. GFP expression in tumor cells results in genetically stable autofluorescence over a long period of time, thereby permitting the quantitative imaging of tumor growth and metastasis formation, as well as their inhibition by agents, such as irinotecan and docetaxel, used in this study. This remarkable experimental animal model has enabled us to describe the contribution of the ceramide/S1P biostat in anticancer therapy, for the first time in vivo.

As a proof of the strategic role of SphK1 in regulating apoptosis of prostate cancer cells, our study showed that cell death caused by chemotherapeutics was markedly inhibited by SphK1 overexpression. These results confirm previous reports that SphK1 overexpression can offer protection against proapoptotic stimuli, namely serum withdrawal, short-chain ceramides, and anthracyclines. Nevertheless, our studies are the first to clearly show that the cytoprotective effect exerted by SphK1-enforced expression is due, as suspected, to a strong down-regulation of the ceramide/S1P biostat. Indeed, as shown here, SphK1 can be inhibited regardless of the cell p53 status. It was reported by Taha et al. (46) that down-regulation of the SphK1 protein occurred in a p53-dependent manner in Molt-4 leukemia cells in response to DNA-damaging agents. The signaling cascades triggered by camptothecins or taxanes are multiple and proteins other than p53 are doubtlessly involved in the regulation of SphK1 activity.

Table 1. Efficacy of irinotecan or docetaxel against metastases

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. mice with metastases/total no. mice</th>
<th>Total no. metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>13/13 (100%)</td>
<td>7, 3, 3, 2, 3, 2, 7, 4, 2, 3, 3, 2, 3 (total: 44)</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>12/12 (100%)</td>
<td>2, 3, 3, 2, 1, 2, 3, 3, 2, 5, 2, 2 (total: 30)*</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>8/12 (66%)*</td>
<td>2, 0, 0, 1, 4, 1, 0, 0, 3, 1, 1 (total: 15)</td>
</tr>
</tbody>
</table>

*P = 0.1289 and P = 0.0068 compared with sham-treated animals, respectively (Wilcoxon rank sum test).
† P = 0.0391 and P = 0.0932 compared with sham-treated and irinotecan-treated animals, respectively (Fisher’s exact test).
‡ P = 0.0163 between camptothecin- and docetaxel-treated animals (Wilcoxon rank sum test).

Figure 6. Orthotopically implanted PC-3-overexpressing SphK1 tumors display stronger resistance to docetaxel. PC-3/Neo or PC-3/SphK1 orthotopic implanted in nude mice were randomized in two groups of six to eight mice each after 3 weeks of growth. Mice were then subjected to three weekly 20 mg/kg docetaxel or sham treatments by IP injections. Tumor mass of excised primary tumor (A), S1P (B), and ceramide content (C). Columns, mean from six to eight animals; bars, SE.
Collectively, these results provide the first in vivo demonstration of SphK1 acting as a cancer therapy sensor. They highlight the therapeutic potential of modulating the ceramide/sphingomyelin pathway. J Clin Invest 2002;110:3–8.

References

1. Oegretan B, Hannon YA. Biologically active sphingo-


16. Itoh M, Kitano T, Watanabe M, et al. Possible role of ceramide as an indicator of chemoresistance: decrease of the ceramide content via activation of glucosylcer-


Sphingosine Kinase-1 as a Chemotherapy Sensor in Prostate Adenocarcinoma Cell and Mouse Models

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