Human T-cell lymphotropic virus type I-transformed T-cells have a partial defect in ceramide synthesis in response to *N*-(4-hydroxyphenyl)retinamide

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Treatment with the synthetic retinoid HPR [*N*-(4-hydroxyphenyl)-retinamide] causes growth arrest and apoptosis in HTLV-I (human T-cell lymphotropic virus type-I)-positive and HTLV-I-negative malignant T-cells [8]. It was observed that HPR-mediated growth inhibition was associated with ceramide accumulation only in HTLV-I-negative cells. The aim of the present study was to investigate the mechanism by which HPR differentially regulates ceramide metabolism in HTLV-I-negative and HTLV-I-positive malignant T-cells. Clinically achievable concentrations of HPR caused early dose-dependent increases in ceramide levels only in HTLV-I-negative cells and preceded HPR-induced growth suppression. HPR induced *de novo* synthesis of ceramide in HTLV-I-negative, but not in HTLV-I-positive, cells. Blocking ceramide glucosylation in HTLV-I-positive cells, which leads to accumulation of endogenous ceramide, rendered these cells more sensitive to HPR. Exogenous cell-permeant ceramides that function par-

tially by generating endogenous ceramide induced growth suppression in all tested malignant lymphocytes, were consistently found to be less effective in HTLV-I-positive cells confirming their defect in *de novo* ceramide synthesis. Owing to its multipotent activities, the HTLV-I-encoded Tax protein was suspected to inhibit ceramide synthesis. Tax-transfected Molt-4 and HELA cells were less sensitive to HPR and C₆-ceramide mediated growth inhibition respectively and produced lower levels of endogenous ceramide. Together, these results indicate that HTLV-I-positive cells are defective in *de novo* synthesis of ceramide and that therapeutic modalities that bypass this defect are more likely to be successful.

Key words: ceramide, human T-cell lymphotropic virus type-I (HTLV-I), *N*-(4-hydroxyphenyl)retinamide (HPR), Tax protein.

INTRODUCTION

The retinoid HPR [*N*-(4-hydroxyphenyl)retinamide] inhibits growth and induces apoptosis in many human cell lines, including many that are all-*trans*-retinoic acid-resistant [1]. HPR's cytotoxicity is mediated through retinoic acid receptor-dependent and -independent mechanisms [2], may involve p53-independent pathways [3,4], is associated with the generation of ROS (reactive oxygen species) [5], is coupled with JNK (c-Jun N-terminal kinase) activation [6] and may require elevated levels of ceramide [7].

We previously reported that at clinically achievable concentrations, HPR is a potent and selective inducer of G₁ cell cycle arrest and apoptosis in HTLV-I-positive and HTLV-I-negative malignant T-cells with no effect on normal T-lymphocytes [8]. In HTLV-I-negative cells only, HPR-induced apoptosis was associated with ceramide accumulation, a sharp decrease in mitochondrial membrane potential, and activation of caspases 8, 9 and 3, and could be partially reverted by the caspase inhibitor z-VAD. In contrast, HTLV-I-positive cells had a slower apoptotic response and required higher HPR concentrations suggesting that Tax protein, or other HTLV-I products, protected infected cells from ceramide accumulation and caspase-mediated apoptosis [8].

Ceramide, a sphingolipid secondary messenger molecule, has been proposed as a co-ordinator of eukaryotic stress responses [9]. Many inducers of stress responses result in ceramide accumulation, usually as a result of sphingomyelin breakdown or by *de novo* synthesis, and sometimes as a result of inhibition of cer-

amide clearance through sphingomyelin synthase or ceramidases [10,11].

Ceramide exerts growth suppressive effects including those on differentiation [12], cell cycle arrest by dephosphorylation of Rb protein [13,14], senescence by inhibiting telomerase [15] and most importantly promotes apoptosis in a variety of cell types [16,17]. Ceramide may induce apoptosis through ROS generation [18], activation of JNK/stress-activated protein kinase [19] and through both caspase-dependent and -independent mechanisms [20]. Inducers of ceramide accumulation include TNF- α (tumour necrosis factor- α), Fas ligand, interleukin-1, γ -interferon, CD28 ligation, complement, serum deprivation, γ -irradiation, heat shock, ultraviolet radiation, and most chemotherapeutic agents examined [21]. Defects in ceramide production render the cells more resistant to killing by these inducers [22–24], while elevation of endogenous ceramide levels lowers the threshold for apoptosis induction by these agents [25,26]. Cell permeant ceramide analogues can also produce the growth suppressive effects of these inducers [27].

Several studies described elevations in ceramide levels following HPR treatment and its role in apoptosis [28]. HL-60 leukaemic cells were first shown to exhibit a transient rise in ceramide levels following HPR treatment. The addition of FB₁ (fumonisins B₁), an inhibitor of *de novo* ceramide synthesis, inhibited HPR-induced apoptosis, while the addition of caspase inhibitors had no effect on ceramide levels suggesting that ceramide synthesis occurs upstream of caspase activation [29].

Abbreviations used: Dh-C₆, dihydro-C₆-ceramide; DMEM, Dulbecco's modified Eagle's medium; DGK, diacylglycerol kinase; FBS, fetal bovine serum; FB₁, fumonisins B₁; GCS, glucosylceramide synthase; HPR, *N*-(4-hydroxyphenyl)retinamide; HTLV-I, human T-cell lymphotropic virus type-I; JNK, c-Jun N-terminal kinase; NF- κ B, nuclear factor- κ B; PDMP, D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; ROS, reactive oxygen species; TNF- α , tumour necrosis factor- α .

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Neuroblastoma cells showed a sustained elevation in ceramide levels after HPR treatment, at concentrations that induced both necrosis and apoptosis, and again FB₁ inhibited apoptosis [4]. This HPR-induced ceramide accumulation in neuroblastoma cell lines was due to the co-ordinated activation of serine palmitoyl-transferase and ceramide synthase in the *de novo* synthesis pathway [30]. As a consequence, combinations of HPR and some modulators of ceramide metabolism may provide a novel chemotherapeutic approach [31]. In previous studies, cytotoxic concentrations of HPR have been shown to increase ceramide levels in acute lymphoblastic leukaemia cell lines, and inhibitors of the ceramide *de novo* pathway abrogated this ceramide accumulation [32]. Furthermore, PC-3 prostate cancer cells, which are relatively resistant to HPR, were rendered much more sensitive by blocking the metabolism of ceramide to glucosylceramide using tamoxifen [33]. All of these studies firmly establish ceramide as an important player in apoptosis induction by HPR.

In this report, we demonstrate that HPR induces distinct ceramide responses in HTLV-I-negative and HTLV-I-positive malignant T-cells. Clinically achievable concentrations of HPR caused early dose-dependent increases in ceramide levels only in HTLV-I-negative cells, which preceded HPR-induced growth suppression. Furthermore, HPR specifically induced *de novo* synthesis of ceramide in these latter cells. Exogenous cell-permeant ceramides induced growth suppression in all malignant lymphocytes tested. Blocking ceramide glucosylation in HTLV-I-positive cells enhanced HPR cytotoxicity in these cells. Tax protein-transfected cells were less sensitive to HPR-mediated growth inhibition and generated lower levels of ceramide.

EXPERIMENTAL

Cell lines and culture conditions

The HTLV-I-transformed CD4⁺ T-cell lines HuT-102, MT-2 and C8166, and the HTLV-I-negative CD4⁺ T-cell lines CEM, Jurkat and Molt-4 were grown as described previously [34]. HeLa cells were grown in DMEM (Dulbecco's modified Eagle's medium). Where indicated, Molt-4, Jurkat, or HeLa cells were transfected with either pSG5-Tax or empty vector using Lipofectamine Plus[®] (Gibco, Invitrogen) according to manufacturer's recommendations.

HPR (Sigma) was prepared as stock solutions in DMSO at 1×10^{-2} M and stored in amber tubes at -80°C . Before HPR addition, cells were cultured for 24 h at 1×10^5 cells/ml in RPMI-1640 medium containing 10% (w/v) FBS (fetal bovine serum) (Gibco, Invitrogen) and antibiotics. All experiments using HPR were performed under yellow light ($\lambda > 500$ nm) to prevent photoisomerization. PDMP (D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol), purchased from Biomol (Plymouth Meeting, PA, U.S.A.), was reconstituted in DMSO at a concentration of 50 mM and was stored at -20°C . PDMP was added to cells, 2 h before HPR treatment, to a final concentration ranging from 10 μM to 30 μM . The final concentration of DMSO never exceeded 0.1% and this concentration showed no effect on the proliferation of all tested cell lines (results not shown). The short-chain cell-permeant ceramides C₂- and C₆-ceramide or C₂- and Dh-C₆ (dihydro-C₆-ceramide) (Biomol) were reconstituted in 100% pure ethanol at a concentration of 40 mM, stored at -20°C and used at final concentrations ranging from 1 μM to 20 μM . Before ceramide and dihydroceramide treatments, cells were cultured in RPMI-1640 medium containing 2% (w/v) FBS. FB₁ (Biomol) was reconstituted in $1 \times \text{PBS}$ at a concentration of 20 mM, stored at -20°C and added to cells at 50 μM final concentration 1 h before HPR treatment.

Growth assays

Cell growth was assessed by cell counts using Trypan Blue dye exclusion protocols and/or the use of the CellTiter 96[®] non-radioactive cell proliferation assay kit (Promega Corp., Madison, WI, U.S.A.) according to the manufacturer's instructions. Cells were grown in 96-well plates (Nunc, Naperville, IL, U.S.A.) and at the initiation of cultures drugs were added at the concentrations and time points indicated. Results are expressed as cell growth relative to that in DMSO-treated controls and are derived from the mean cell growth of quadruplicate wells. Results are representative of at least three independent experiments. Similar growth trends were observed using the Trypan Blue dye exclusion and the CellTiter 96[®] non-radioactive cell proliferation assays.

Ceramide measurement

Lipids were collected by the method of Bligh and Dyer [35]. Ceramide was measured with a modified DGK (diacylglycerol kinase) assay using external ceramide standards as described previously [36]. Briefly, 80% of the lipid sample was dried under N₂. The dried lipid was solubilized in 20 μl of an octyl β -D-glucoside/dioleoyl phosphatidylglycerol micellar solution (7.5% octyl β -D-glucoside, 25 mM dioleoyl phosphatidylglycerol) by several cycles of sonication in a bath sonicator followed by resting at room temperature for 15–20 min. The reaction buffer was prepared as a 2 $\times$ solution, containing 100 mM imidazole HCl (pH 6.6), 100 mM LiCl, 25 mM MgCl₂ and 2 mM EGTA. To the lipid micelles, 50 μl of 2 $\times$ reaction buffer were added, 0.2 μl of 1 M dithiothreitol, 5 μg of diglycerol kinase membranes and dilution buffer (10 mM imidazole, pH 6.6, 1 mM diethylenetriamine-penta-acetic acid, pH 7) to a final volume of 90 μl . The reaction was started by adding 10 μl of 2.5 mM [γ -³²P]ATP solution (specific activity of 75 000–200 000 c.p.m./nmol). The reaction was allowed to proceed at 25°C for 30 min. Bligh and Dyer lipid extraction was performed and a 1.5 ml aliquot of the organic phase was dried under N₂. Lipids were then resuspended in 50 μl of methanol/chloroform (1:9, v/v) and 25 μl was spotted on to a 20 cm silica gel TLC plate. Plates were developed using chloroform/acetone/methanol/acetic acid/H₂O (50:20:15:10:5, by vol), air-dried and subjected to autoradiography. The radioactive spots corresponding to phosphatidic acid and ceramide-phosphate, the phosphorylated products of diacylglycerol and ceramide respectively, were identified by comparison with known standards. Spots were scraped into a scintillation vial containing 4 ml of scintillation fluid and counted on a scintillation counter. Linear curves of phosphorylation were produced over a concentration range of 0–960 pM of external standards (dioleoyl glycerol and CIII-ceramide, Sigma). Ceramide levels were routinely normalized to lipid phosphate levels. It is important to note that under these conditions, there was a total conversion of ceramide and diacylglycerol into their phosphorylated products, and there was no change in the specific activity of the DGK enzyme.

De novo ceramide synthesis

At initiation of treatment, [³H]palmitic acid (1 $\mu\text{Ci}/\text{ml}$ medium), purchased from PerkinElmer (Boston, MA, U.S.A.) was added to HPR-treated and control samples. Extracted lipids, dried under N₂, were resuspended in 1 ml of chloroform, 200 μl was set aside for the first phosphate measurement and 800 μl was used in the base hydrolysis assay. In this assay, 200 μl of chloroform and 800 μl of 0.125 M methanolic KOH were added to the samples before incubation at 37°C for 75 min. The base was neutralized by adding 100 μl of 1 M methanolic HCl to each sample. A 2 ml volume of chloroform and 600 μl of distilled H₂O

were next added to the samples at room temperature for 30 min. Subsequently, lipids were extracted by the method of Bligh and Dyer [35], dried under N₂ and resuspended in 75 μ l of chloroform. A 50 μ l sample of hydrolysed lipids was subjected to separation for 1 h on a 20 cm silica gel TLC plate and 25 μ l was kept for a second phosphate assay. The solvent system employed in TLC separation was chloroform/methanol/2 M NH₄OH (4:1:0.1, by vol). The TLC plates were then sprayed lightly with En³hance[®] (PerkinElmer) to enhance tritium readings. The ceramide spots were visualized by iodine vapour mark. Radioactivity was also visualized by autoradiography after 48 and 96 h at 80 °C and the [³H]ceramide spots were scraped into scintillation vials containing 4 ml of scintillation fluid and counted on a Packard scintillation counter. Ceramide was quantified using external standards and was normalized to phosphate counts.

Treatment with 17C₆-ceramide

Hela cells, seeded at 2×10^6 cells/ml, were transfected with either pSG5-Tax or empty vector using Lipofectamine Plus[®] (Gibco) according to the manufacturer's recommendations. At 24 h post-transfection, cells were treated with D-erythro-17C₆-ceramide (generously provided by Dr Alicia Bielawska from the Lipidomics Core at the Medical University of South Carolina, Charleston, South Carolina, U.S.A.) for a further 24 h. This unnatural ceramide undergoes deacylation and reacylation shortly after cell entry where the generated 17-sphingosine backbone is recycled and the activity of different ceramide synthases results in the formation of several species of endogenous 17-ceramide. Cell pellets were spiked with internal standards, lipids were extracted and the unnatural 17-ceramide molecular species reflecting the activity of ceramide synthases were analysed by MS. Data were normalized to total protein levels.

Microsome preparation

Microsomes were prepared by sonication of cell pellets from CEM and HuT-102 cells in 25 mM Tris, pH 7.4, 5 mM EDTA, 1 mM PMSF and 1 $\times$ complete protease inhibitor (Roche, Mannheim, Germany)-containing buffer. The preparation was centrifuged at 1000 g, and the resulting supernatant was then ultracentrifuged at 100 000 g. The pellet was resuspended by homogenization in Hepes buffer (20 mM Hepes, 2 mM KCl and 2 mM MgCl₂) at pH 7.4. Protein concentration was determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

In vitro ceramide synthase activity assay

Ceramide synthase activity was determined using microsome fractions isolated from HPR-treated and control cells. A 50 μ g sample of microsomal proteins was incubated in 25 mM potassium phosphate, pH 7.4, containing 50 μ M palmitoyl-CoA and 15 μ M D-erythro-17-sphingosine (a kind gift from Dr Alicia Bielawska) delivered using BSA as vehicle. The incubation was performed for 15 min at 37 °C and was stopped by the addition of the extraction solvent. Extracted lipids were used to measure the levels of 17C₁₆-ceramide by MS.

MS analysis of lipids

Samples were fortified with internal standards and lipids were extracted with ethyl acetate/isopropanol/water (60:30:10 by vol), evaporated to dryness and reconstituted in 100 μ l of methanol. Analysis was performed using electrospray ionization MS/MS analysis on a Thermo Finnigan TSQ 7000 triple quadrupole

mass spectrometer, operating in multiple-reactions-monitoring positive-ionization mode, as described previously [37].

Statistical analysis

Three statistical tests were performed to validate the significance of the observed results: the Dunnett test, the two-way analysis of variance test, and the two-sample Students *t*-test.

RESULTS

HPR induces distinct ceramide responses in HTLV-I-negative and HTLV-I-positive malignant T-cells

The effects of HPR treatment on ceramide levels was examined in HTLV-I-negative cells. A time-dependent increase in ceramide was observed in CEM (Figure 1A), Jurkat and Molt-4 cells (results not shown) treated with HPR. This increase in ceramide levels started after as little as 6 h in Jurkat cells and 12 h in CEM cells, reaching a 2-fold increase after 18 h and increased to at least 3-fold after 24 h in both cell lines. Ceramide generation preceded the significant HPR-induced growth suppression and cell death observed in these cell lines at the designated concentrations (Figure 1B). A dose-dependent accumulation of ceramide was noted in CEM cells treated for 24 h with increasing concentrations of HPR, ranging from 0.1 μ M to 5 μ M (Figure 1C). In contrast, no increase in ceramide levels was observed in HTLV-I-positive HuT-102 (Figure 1D), C8166, and MT-2 cells (results not shown) after 24 h of HPR treatment at 5 μ M despite significant growth suppression and cell death observed under these conditions [8]. Only late, modest increases in ceramide levels were observed in HTLV-I-positive cells treated with 5 μ M HPR for 48 h ($P < 0.01$, *t*-test) (Figure 1D), after significant cell death had occurred [8]. Also, lower HPR doses had no effect ($P < 0.01$, Dunnett test) (Figure 1E).

HPR induces *de novo* synthesis of ceramide in HTLV-I-negative, but not in HTLV-I-positive, malignant T-cells

Previous studies in different cell lines indicated that HPR induces *de novo* synthesis of ceramide [30–32]. We examined the role of *de novo* ceramide synthesis following HPR treatment of HTLV-I-negative and HTLV-I-positive cell lines. CEM and C8166 cells were treated with 1 and 5 μ M HPR concentrations respectively, based on their differential HPR sensitivity [8] at indicated time points, and *de novo* synthesized ceramide was measured by [³H]palmitate incorporation. HPR treatment caused a time-dependent increase in [³H]ceramide, indicating *de novo* synthesis in CEM cells, starting at 18 h post-treatment ($P < 0.01$, Dunnett test) (Figure 2). In contrast, C8166 cells showed no change in [³H]ceramide after treatment using a 5-fold higher HPR concentration (Figure 2). The activity of ceramide synthase in CEM and HuT-102 cells in response to HPR treatment was 129.1 ± 5.4 and 89.3 ± 2.8 pmol of ceramide/min per mg of protein respectively. There was no significant induction of this activity when compared to the corresponding untreated cells. These results suggest that *de novo* ceramide synthesis was impaired in HTLV-I-infected cells.

Inhibition of GCS (glucosylceramide synthase) increases sensitivity to HPR-induced cell death in HTLV-I-positive cells

We next aimed to determine whether increasing the endogenous levels of ceramide in HTLV-I-positive cells renders them more sensitive to HPR. We therefore investigated the effect of PDMP, an

Figure 1 HPR induces distinct ceramide responses in HTLV-I-negative and HTLV-I-positive malignant human T-cell lines

(A) Ceramide levels in the HTLV-I-negative CEM human T-cell line in response to HPR. CEM cells were seeded at a density of 3.5×10^5 cells/ml and treated with 0.1% DMSO as a control or with 1 μ M HPR for the times indicated. Ceramide levels were determined using the DGK assay as described in the Experimental section and normalized to total cellular lipid phosphate levels. Data points represent the mean $\pm$ range ($n=2$). Results are representative of three independent experiments. (B) Effects of HPR treatment on the growth of CEM human T-cell line. Cells were seeded at a density of 2×10^5 cells/ml and treated with 0.1% DMSO as a control or with 1 μ M HPR for the times indicated. Viable cell counts, calculated from triplicate wells by Trypan Blue dye exclusion, are expressed as a percentage of controls and are representative of two independent experiments. (C) Dose-response to HPR treatment in CEM human T-cell line. Cells were seeded at a density of 3.5×10^5 cells/ml and treated with 0.1% DMSO as a control, or the indicated concentrations of HPR, for 24 h. Ceramide levels were determined as in (A). Data points represent the mean $\pm$ range ($n=2$). Results are representative of three independent experiments. (D) HPR treatment induces late ceramide accumulation in HTLV-I-positive human T-cell line Hut-102. Cells were seeded at a density of 3.5×10^5 cells/ml and treated with 0.1% DMSO as a control or 5 μ M HPR for the times indicated. Ceramide levels were determined as in (A). Data points represent the mean $\pm$ range ($n=2$). Results are representative of two independent experiments. (E) Dose-response to HPR treatment in HTLV-I-positive human T-cell line C8166. Cells were seeded at a density of 3.5×10^5 cells/ml and treated with the indicated concentrations of HPR for 48 h. Ceramide levels were determined as in (A). Data points represent the mean $\pm$ range ($n=2$). Results are representative of two independent experiments.

Figure 2 HPR treatment induces *de novo* synthesis of ceramide in HTLV-I-negative human T-cell lines only

CEM and C8166 cells were seeded at a density of 3.5×10^5 cells/ml and treated with 0.1% DMSO as a control or with 1 and 5 μ M HPR respectively. *De novo* ceramide levels were determined using the [3 H]palmitate incorporation method as described in the Experimental section and normalized to total cellular lipid phosphate levels. Data points represent the mean $\pm$ range ($n=2$) and are percentages of treated over control cells. Results are representative of two independent experiments.

inhibitor of GCS [38] and thus an inducer of ceramide accumulation, on HPR-induced cytotoxicity. At 2 h before HPR exposure, cells were pretreated with PDMP at non-cytotoxic concentrations: 20 μ M in C8166 and Hut-102 and 30 μ M in MT2 cells. These concentrations resulted in a modest elevation of cellular ceramide levels (Figure 3A, and results not shown). The combination of HPR and PDMP in HTLV-I-positive cells resulted in a reduction in cell viability ($P < 0.01$, *t*-test) exceeding their additive effects (Figure 3B). Treatment with 1 μ M HPR and 20 μ M PDMP for 24 h led to a 12% and 16% synergistic reduction in cell viability in C8166 and Hut-102 cells respectively ($P < 0.01$, *t*-test) (Figure 3B). Because of their lower sensitivity to both PDMP and HPR [8], MT2 cells were treated with

3 μ M HPR and 30 μ M PDMP for 24 h, resulting in a significant 21% synergistic reduction in cell viability ($P < 0.01$, *t*-test) (results not shown). Only additive effects of HPR and PDMP treatments were observed in the HTLV-I-negative cell line CEM, although ceramide levels increased by approx. 100% over control in response to PDMP (results not shown). These results indicate that increasing endogenous ceramide levels by PDMP partially restores the sensitivity of HTLV-I-positive cells to HPR treatment.

Exogenous ceramides induce cell death in HTLV-I-negative and HTLV-I-positive malignant T-cells

The results above suggested that HTLV-I-positive cells may have a defect in ceramide synthesis in response to HPR and that elevation of endogenous ceramide by PDMP renders them more sensitive to HPR. Therefore it became important to determine the sensitivity of both HTLV-I-positive and HTLV-I-negative cells to exogenous ceramide as this might bypass the defect. Addition of cell-permeant C_2 - and C_6 -ceramide analogues triggers many of the biological responses of agonists in cells [22]. We treated the HTLV-I-negative Jurkat cells and the HTLV-I-positive Hut-102, MT2 and C8166 cells with increasing concentrations of C_2 -ceramide, ranging from 1 to 20 μ M for up to 3 days. In all cells tested, C_2 -ceramide caused a reduction in their viability in a time- and dose-dependent manner (Table 1). A 20 μ M C_2 -ceramide concentration completely killed the majority of tested cells by 24 h. When the effects of the same concentration of C_2 -ceramide in both cell type groups were compared, HTLV-I-positive cells were slightly more resistant than HTLV-I-negative cells. Additionally, cell treatment with Dh- C_6 , the immediate inactive precursor of ceramide, ranging from 1 to 20 μ M for up to 3 days, showed no effect on cell viability in any cell line, underscoring the specificity of ceramide action (Table 1).

Figure 3 PDMP synergizes with HPR treatment to induce cell death in HTLV-I-positive human T-cell lines

(A) Ceramide levels in the HTLV-I-positive C8166 and HuT-102 cell lines in response to combined HPR and PDMP treatment. Cells were seeded at a density of 3.5×10^5 cells/ml and were pretreated with 20 μ M PDMP, an inhibitor of GCS. PDMP pretreatment was performed 2 h before treatment with 1 μ M HPR. Data points represent the mean $\pm$ range ($n=2$). Results are representative of two independent experiments. (B) Effect of combined HPR and PDMP treatment on the growth of the HTLV-I-positive C8166 and HuT-102 cells. Cells were seeded at a density of 2×10^5 cells/ml and were pretreated with 20 μ M PDMP, an inhibitor of GCS. PDMP pretreatment was performed 2 h prior to treatment with 1 μ M HPR. Viability was assayed in 96-well plates at 24 h using the Cell Titer 96[®] non-radioactive cell proliferation kit. Data points represent the mean $\pm$ S.D. ($n=3$). Results are expressed as percentage of control cells (0.1% DMSO) and represent the mean of three independent experiments.

Recently, C₆-ceramides were shown to induce the generation of endogenous long-chain ceramides [39]. Our previous results demonstrated that the HTLV-I-positive cells are not capable of HPR-induced *de novo* ceramide synthesis and therefore may be relatively less sensitive to C₆-ceramides than the HTLV-I-negative cells. We treated several HTLV-I-positive HuT-102, MT2 and C8166 cells and HTLV-I-negative Jurkat and CEM cells with C₆-ceramide, ranging from 1 to 20 μ M for up to 3 days. C₆-ceramide caused a time- and dose-dependent reduction in the growth of all cells tested (Table 1). Viability of HTLV-I-positive-C8166 cells was measured using the CellTiter 96[®] non-radioactive cell proliferation assay owing to excessive clumping of cells observed in this cell line. In response to 20 μ M C₂-ceramide, viability decreased to 49%, 2% and 0% of control at 24, 48 and 72 h after treatment respectively. In response to the same concentration of C₆-ceramide, viability decreased to 69%, 23% and 18% at the same respective time points. Interestingly, when the effects of the same concentration of C₆-ceramide were compared in both cell type groups, the HTLV-I-negative cells were found to be significantly more sensitive to C₆-ceramide than HTLV-I-positive cells (Table 1). These results suggested, that in response to C₆-ceramide, HTLV-I-positive cells might generate lower levels of endogenous ceramides.

Response to C₆-ceramide in HTLV-I-positive cells is blunted, resulting from a defect in endogenous ceramide synthesis

In order to verify whether ceramide synthesis in response to exogenous C₆-ceramide is affected in HTLV-I-positive cells, we treated HuT-102, MT2 and C8166 cells, and the control CEM, Jurkat and Molt-4 cells with 20 μ M C₆-ceramide. Ceramide levels were measured using the DGK assay, where the phosphorylated products of C₆-ceramide and endogenous ceramide can be identified by their distinct migration. C₆-ceramide induced significantly lower endogenous ceramide accumulation after 12 (results not shown) and 18 h (Figure 4A) in all HTLV-I-positive cells tested, compared with HTLV-I-negative cells. Elevated endogenous ceramide levels in HTLV-I-negative cells correlated with higher levels of growth suppression and cell death in these cells after 12 (results not shown) and 18 h (Figure 4B) as compared with HTLV-I-positive cells (C8166 viability decreased to 74% of

Table 1 Exogenous ceramides induce cell death in HTLV-I-negative and HTLV-I-positive malignant human T-cell lines

Effects of synthetic ceramides C₂ and C₆ on the growth of the HTLV-I-negative (Jurkat) and the HTLV-I-positive (HuT-102 and MT2) human T-cell lines. Cells were seeded in RPMI 1640 medium containing 2% (w/v) FBS in 24-well plates at a density of 2×10^5 cells/ml and were treated with the indicated concentrations of C₂-ceramide, C₆-ceramide and Dh-C₆. Viability was assayed using the Trypan Blue dye exclusion method. Data points represent the mean viability ($n=3$). Results are expressed as percentage of control cells (0.1% ethanol) and represent the mean of three independent experiments.

Cells	Ceramide (μ M)	Treatment time (h)								
		24			48			72		
		C ₂	C ₆	Dh-C ₆	C ₂	C ₆	Dh-C ₆	C ₂	C ₆	Dh-C ₆
JURKAT	1 μ M	90	90	100	62	80	100	83	100	99
	5 μ M	87	95	99	38	65	98	24	70	96
	10 μ M	40	82	98	11	61	97	3	55	95
	20 μ M	0	0	95	0	0	95	0	0	94
HuT-102	1 μ M	92	94	100	67	65	100	56	100	100
	5 μ M	55	60	98	38	56	98	29	92	96
	10 μ M	31	51	97	10	42	96	0	78	94
	20 μ M	8	42	95	0	35	94	0	52	94
MT2	10 μ M	81	93	97	79	80	95	91	64	95
	15 μ M	32	70	96	20	64	95	13	58	95
	20 μ M	12	65	96	1	39	94	0	25	94

Figure 4 Response to exogenous C_6 -ceramide in HTLV-I-positive human T-cell lines is blunted because of a defect in endogenous ceramide synthesis

(A) Endogenous ceramide levels in the HTLV-I-positive (HuT-102, MT2 and C8166) and the HTLV-I-negative (CEM, Jurkat and Molt-4) human T-cell lines in response to C_6 -ceramide. Cells were seeded in RPMI-1640 medium containing 2% FBS at a density of 3.5×10^5 cell/ml. Cells were treated with $20 \mu\text{M}$ C_6 -ceramide for 18 h. Endogenous ceramide levels were determined as in Figure 1(A). The phosphorylated product of C_6 -ceramide is easily distinguishable from that of endogenous ceramide on the TLC plate (results not shown). Data points represent the mean $\pm$ range ($n = 2$). Results are representative of two independent experiments. (B) Effects of C_6 -ceramide on the growth of the HTLV-I-positive (HuT-102 and MT2) and the HTLV-I-negative (CEM, Jurkat and Molt-4) human T-cell lines. Cells were seeded in RPMI-1640 medium containing 2% FBS at a density of 2×10^5 cells/ml. Cells were treated with $20 \mu\text{M}$ C_6 -ceramide for 18 h. Viability was assayed using the Trypan Blue dye exclusion method. Data points represent the mean $\pm$ S.D. ($n = 3$). Results are expressed as percentage of control cells (0.1% ethanol) and represent the mean of two independent experiments. (C) Endogenous ceramide levels in the HTLV-I-positive HuT-102 and the HTLV-I-negative Jurkat human T-cell lines in response to C_6 -ceramide and FB_1 treatment. Cells were seeded in RPMI-1640 medium containing 2% FBS at a density of 3.5×10^5 cell/ml. Cells were treated with $20 \mu\text{M}$ C_6 -ceramide, $50 \mu\text{M}$ FB_1 , or combined C_6 -ceramide and FB_1 for 18 h. Endogenous ceramide levels were determined as in Figure 1(A). Data points represent the mean $\pm$ range ($n = 2$). Results are representative of two independent experiments. (D) Endogenous ceramide levels in the HTLV-I-positive HuT-102 and the HTLV-I-negative Jurkat human T-cell lines in response to exogenous C_2 -ceramide. Cells were seeded in RPMI-1640 medium containing 2% FBS at a density of 3.5×10^5 cell/ml. Cells were treated with $20 \mu\text{M}$ C_2 -ceramide for 18 h. Endogenous ceramide levels were determined as in Figure 1(A). Data points represent the mean $\pm$ range ($n = 2$). Results are representative of two independent experiments.

controls as measured using the CellTiter 96[®] non-radioactive cell proliferation assay kit). To investigate the role of the *de novo* ceramide synthetic pathway upon exogenous C_6 -ceramide addition, HuT-102 and Jurkat cells were pretreated with FB_1 , an inhibitor of ceramide synthase. The addition of FB_1 almost completely abrogated the endogenous ceramide levels that were generated at 12 and 18 h after C_6 -ceramide addition in both cell lines (Figure 4C). The viability of HPR-treated Jurkat and HuT-102 cells for up to 12 h increased by 35% and 14% respectively, following FB_1 pre-treatment, suggesting that endogenous ceramide accumulation contributes significantly to HPR-induced cell death of HTLV-I-negative cells.

The more potent growth suppressive effects of C_2 -ceramide compared to C_6 -ceramide in both HTLV-I-positive and HTLV-I-negative cells (Table 1) led us to examine whether the activity of this analogue was less dependent on endogenous ceramide generation. We treated HuT-102 and Jurkat cells with $20 \mu\text{M}$ C_2 -ceramide to evaluate its ability to induce endogenous ceramide synthesis. Indeed, lower levels of endogenous ceramide levels accumulated in the HTLV-I-positive cells after C_2 -ceramide administration versus C_6 -ceramide (compare Figure 4D with Fig-

ure 4C), suggesting an explanation for the improved activity of this analogue in HTLV-I-positive cells. Taken together, these results clearly demonstrate that the HTLV-I-positive cells are defective in endogenous ceramide synthesis, not only in response to HPR, but also in response to exogenous ceramides.

Tax protein represses the ceramide response in HTLV-I-positive cells

In order to test whether, in HTLV-I-infected cells, the oncoprotein Tax is responsible for inhibiting ceramide accumulation, we treated Tax-transfected Molt-4 cells with HPR (5 and $10 \mu\text{M}$) and measured ceramide levels. The levels of ceramide generated were significantly lower in Tax-transfected cells in response to both HPR concentrations after 14 and 18 h ($P < 0.05$, *t*-test) (Figure 5A). However, $5 \mu\text{M}$ HPR treatment for 20 and 38 h in the HTLV-I-negative CEM cells and HTLV-I-positive HuT-102 cells did not significantly stimulate ceramide synthase activity (results not shown). Furthermore, HeLa cells transiently transfected with Tax protein had a significantly reduced accumulation of endogenous ceramide after 18 h (Figure 5B) in response to

Figure 5 Role of Tax protein in suppressing the ceramide response in the HTLV-I-positive human T-cell lines

(A) Reduced ceramide levels in HPR-treated Tax-transfected Molt-4 cells. Molt-4 cells were transiently transfected with pSG5-Tax or control vector and treated after 24 h with HPR (5 μ M and 10 μ M) for the times indicated. Ceramide levels were determined as in Figure 1(A). Data points represent the mean $\pm$ range ($n=2$). Results are representative of two independent experiments. (B) Effects of exogenous C_6 -ceramide on endogenous ceramide levels in Tax-transfected HeLa cells. pSG5-Tax or control cells were transfected for 24 h and then were treated with 20 μ M C_6 -ceramide in DMEM medium containing 2% FBS for 18 h. Endogenous ceramide levels were determined as in Figure 1(A). Data points represent the mean $\pm$ range ($n=2$). Results are representative of two independent experiments. (C) Ceramide synthase activity is reduced in Tax-transfected HeLa cells. pSG5-Tax or control cells were transfected for 24 h and then were treated with 5 μ M 17C₆-ceramide in DMEM medium containing 2% FBS for 24 h. Endogenous 17-ceramide species levels were determined as described in the Experimental section and normalized to total cellular protein levels. Data points represent the mean $\pm$ range ($n=2$).

C_6 -ceramide treatment ($P < 0.01$, t -test). Moreover, we utilized the synthetic ceramide 17C₆-ceramide, that has an unnatural 17-sphingosine backbone, to indirectly determine the activity of ceramide synthase(s). Measurement of the unnatural endogenous 17-ceramides that result from the deacylation/reacylation reaction (Figure 5C) after 24 h showed that in Tax-transfected cells, the production of most endogenous 17-ceramides was significantly inhibited ($P < 0.01$ for all except 17C₂₄-ceramide, t -test). These

experiments when combined with the results above indicate that Tax is responsible for suppression of *de novo* ceramide synthesis, probably by inhibiting ceramide synthase.

DISCUSSION

We have determined that HPR induces distinct ceramide responses in HTLV-I-negative and HTLV-I-positive malignant T-cells. This may be partly explained by an impaired *de novo* ceramide synthetic pathway in the HTLV-I-positive cells owing to expression of Tax protein. *De novo* synthesis of ceramide is dependent on the activity of several enzymes, including several ceramide synthases. Recently, ceramide synthase genes, identified as Lass (longevity assurance-like) genes, were described but have not yet been well characterized [40]. The results from this study indicate that the activity of ceramide synthase(s) is suppressed in HTLV-I-positive cells.

The HTLV-I-encoded oncoprotein, Tax, plays a key role in the transformation of infected cells and their resistance to chemotherapy [8,41]. In the current study, it is shown that Tax expression in cells not infected with HTLV-I is sufficient to inhibit the generation of ceramide in response to HPR or C_6 -ceramide and to suppress ceramide synthase activity as measured by the ability of transfected cells to convert the 17C₆-ceramide into endogenous 17-ceramides. A major biological function of Tax protein appears to be its ability to induce a constitutive high level of activity of the transcription factor NF- κ B (nuclear factor- κ B) [42]. Recent evidence suggests that the Tax oncoprotein represses the transcriptional activity of the tumour suppressor protein p53 through the activation of the NF- κ B pathway, but independent of the CREB/ATF pathway, which is also disrupted by Tax [43]. Interestingly, p53 was found to regulate the generation of ceramide in response to chemotherapeutic agents or γ -irradiation [44]. Therefore one future aim will be to examine whether NF- κ B and p53 mediate the inhibitory effects of Tax on *de novo* synthesis of ceramide at the genetic level.

Cell permeant ceramide analogues C2 and C6 were shown to reproduce the growth suppressive effects of chemotherapeutic agents that induce ceramide accumulation [27]. These short-chain ceramides have also been employed to support evidence for apoptosis induction obtained after elevation of cellular ceramide content in response to several inducers of ceramide accumulation such as TNF- α , Fas ligand, interleukin-1 and other effectors [45–47]. C_6 -ceramide was recently shown to induce *de novo* ceramide synthesis in the A549 human lung adenocarcinoma cell line where its sphingosine backbone becomes recycled after deacylation/reacylation into endogenous long-chain ceramides [39]. This revealed that the biological functioning of exogenous ceramides was in part dependent on ceramide synthase activity to produce endogenous ceramides. In the current study C₂- as well as C_6 -ceramides induced *de novo* ceramide synthesis in both HTLV-I-positive and HTLV-I-negative cells. However, this was markedly lower in HTLV-I-positive cells, and in both cell types, was lower in response to the C₂-ceramide analogue. Both analogues induced growth suppression in both cell types in a time- and dose-dependent manner. The lower sensitivity of HTLV-I-positive cells to exogenous ceramides provided further support for the suggestion that ceramide synthase is inhibited in these cells. It was also noted, that under the same conditions, C₂-ceramide was more effective compared with C_6 -ceramide in producing growth suppression. These differences could be due to better uptake, slower metabolism, or an enhanced direct effect for this analogue, as opposed to dependence on the generation of endogenous ceramide.

Several studies indicate that the co-administration of modulators of ceramide metabolism and chemotherapeutic agents increases the level of ceramide and further enhances cytotoxicity. PDMP and related compounds are potent inhibitors of GCS and consequently of ceramide clearance. These inhibitors have been shown to sensitize a variety of drug-resistant cell lines to chemotherapeutic reagents and cause their preferential killing by such reagents [26,48,49]. Furthermore, D,L-threo-(1-phenyl-2-hexadecanoylamino)-3-morpholino-1-propanol administration produces a synergistic increase in cytotoxicity with HPR in solid tumour cell lines [31] and in HPR-resistant PC-3 prostate cancer cells [33]. In the present study HTLV-I-positive cells were found to have a defect in ceramide synthesis and relative resistance to HPR as compared with HTLV-I-negative cells. Restoration of elevated ceramide levels by PDMP during HPR treatment led to synergistic cytotoxicity in HTLV-I-positive cell lines, thus sensitizing these cells to HPR concentrations that are lower than what is usually required to induce significant growth suppression. This, together with results using exogenous ceramides, indicated that HTLV-I-positive cells were sensitive to ceramide-induced cell killing once sufficient levels were generated.

Deregulation of ceramide metabolism may act as an indicator of chemoresistance in tumour cells and could be a target in cancer therapy [28,50]. The present study demonstrates that viral oncogenes may target the ceramide pathway. In the case of Tax, this occurs by inhibition of ceramide synthase activity and may explain the resistance of HTLV-I-positive cells to treatment. Therapeutic agents with the ability to bypass this defect are more likely to be successful.

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