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Programmed cell death in neurotumour cells involves the generation of ceramide*

DOUGLAS A. WIESNER[1] and GLYN DAWSON[1,2]‡

Department of [1]Pediatrics MC4068,
[2]Biochemistry and Molecular Biology, University of Chicago School of Medicine, 5841 S. Maryland Avenue, Chicago IL 60637, USA

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Ceramide has been typically thought of as the membrane anchor for the carbohydrate in glycosphingolipids but many studies have suggested that it may cause apoptosis. Apoptosis or programmed cell death (PCD) is thought to be responsible for the death of one-half of neurons surviving the development of the nervous system. The potential involvement of the sphingomyelin-ceramide signaling process as an integral part of PCD was therefore examined in several neurotumour cell lines. We show that synthetic C2-ceramide (*N*-acetylsphingosine), a soluble ceramide analogue, can rapidly trigger PCD in these cells, characterized by: 1) classic DNA laddering on agarose gels; 2) DNA fragmentation as determined by Hoechst Dye; and 3) cell viability (mitochondrial function and intact nuclei) assays. We report that staurosporine can both activate PCD (by all three criteria above) in neurotumour cells and increase both the formation of ceramide and ceramide mass. Both ceramide formation and the induction of PCD were further enhanced by the co-addition of a ceramidase inhibitor oleoylethanolamine (25 μM). Staurosporine and oleoylethanolamine were similarly effective in inducing ceramide formation and PCD in immortalized hippocampal neurons (HN-2) and immortalized dorsal root ganglion cells (F-11). Our data suggests that formation of ceramide is a key event in the induction of PCD in neuronally derived neurotumour cells.

Keywords: programmed cell death, apoptosis, neurons, immortalized neurons, ceramide, staurosporine, oleoylethanolamine

Abbreviations: PCD: programmed cell death; PKC: protein kinase C; HPTLC: high-performance thin-layer chromatography; DETAPAC: diethylenetriaminepentaacetic acid; DMEM: Dubelco's modified Eagle's medium; FCS: fetal calf serum; PBS: phosphate-buffered saline; DAG: diacylglycerol; DDI: distilled-deionized; Cer: ceramide; SM: sphingomyelin.

Introduction

Glycosphingolipids have long been associated with oncogenic transformation [1], however, it has always been assumed that it was the oligosaccharide head group that was important for biological function. Recently there have been several studies that have implicated the backbone of sphingolipids, ceramide, as having significant biological function as a lipid signalling molecule in programmed cell death (PCD).

Apoptosis, or PCD, is characterized by shrinking of cells, chromatin condensation leading to DNA fragmentation, and the formation of apoptotic bodies coated with phosphatidylserine which are then engulfed by macrophages [2]. This is a natural physiological process that is thought to be responsible for the death of one-half of neurons during the development of the nervous system. Apoptosis has also been implicated in clonal deletion and cell kill by cytokines used by the immune system through the activation of the sphingomyelin pathway. In HL-60 cells, TNF-α activates a putative surface neutral sphingomyelinase which hydrolyses sphingomyelin to ceramide and phosphorylcholine [3–5]. Ceramide is then internalized and down-stream events are only partially known but may involve a proline-directed 97 kDa kinase [6], a ceramide-activated protein phosphatase or a phosphatase and then kinases [3] or PKC ζ [7–9] or leading to

*Dedicated to Dr Sen-itiroh Hakomori in celebration of his 65th birthday.
‡To whom correspondence should be addressed.

activation of ICE-like proteases, endonucleases and DNA disintegration into ~200 bp fragments. The mechanism of activation of transmembrane, neutral, Mg^{2+}-dependent sphingomyelinase is largely unknown, although arachidonic acid has been proposed as an activator [10].

PCD is a well characterized developmental phenomenon that removes unwanted and potentially hazardous cells without complications of inflammation. Deregulated PCD has been suggested to aid in the progression of cancer cells by allowing mutated cells to grow unchecked. There is a large body of work examining the 'neurotrophic' theory of developmental cell death in which an excess of sympathetic neurons compete for growth factors for survival [11]. Neurons not making the correct connections do not receive the appropriate growth factor, and thus die through apoptosis, ensuring the correct number and placement of neurons in the PNS. However, little work has been done to study transformed cells and how they have escaped their requirement for growth factors and by what mechanism the death programme can be restarted as a defence mechanism in the battle against cancer.

In this report, we examine the role of the sphingomyelin-ceramide pathway in programmed cell death in neurotumour cells.

Materials and methods

CHEMICALS

C2-Ceramide and oleoylethanolamine were obtained from Matreya Inc., Pleasant Gap, PA. and staurosporine and authentic ceramides from Sigma. All other chemicals were obtained from Sigma unless mentioned. Palmitic acid, [9,10-^{3}H(N)] (39 Ci mmol^{-1}) and ATP[γ-32P] was purchased from NEN, Boston, MA, En^{3}Hance from Dupont, Xomat-AR film from KODAK and LMP-K HPTLC plates from Whatman, Inc. Sphingomyelinase (*Staphylococcus aureus*) was from Sigma and diacylglycerol kinase (*Escherichia coli*) was from Calbiochem.

CELL CULTURE

Cell cultures of HN-2 (a mouse hippocampal cell × N18TG2 neuroblastoma cell line [12]), and F-11 (a mouse N18TG2 neuroblastoma × rat dorsal root ganglion sensory neuron hybrid cell line [13]), were maintained in Dubelco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum and 1% gentamicin. For experiments, cells were incubated in serum free DMEM prior to use. Ceramide, oleoylethanolamine and staurosporine were added in DMSO.

Cell viability assay

The modified MTT assay [14] was used in conjunction with Coulter counting of cell nuclei. The MTT assays were done in 24-well culture dishes containing 250 µl of media. After treatment, 25 µl of stock MTT (5 mg ml^{-1} in sterile PBS) was added and incubated for 15 min at 37 °C. Finally, a solution of 250 µl of 10% SDS in 0.01 M HCl was added. After incubating overnight, the absorption value at 570 nm was determined with a Hitachi spectrophotometer. Viability was determined as % survival = [(exp–blank)/(control–blank)] where exp is the reading for the treated cells, control is untreated cells, and blank is MTT added just to the media.

DNA ANALYSES

DNA extraction and electrophoresis

Cells were cultured in 60 mm dishes in 10% fetal calf serum supplemented DMEM prior to treatment. When stated, the media was replaced with serum free and, after 1 h recovery time, the cells treated with the specified drug for the stated time. Cells were washed with ice-cold PBS and lysed in 0.5 ml of 0.6% SDS, 10 mM EDTA, pH 7.0. Sodium chloride (1 M) was added to the lysate and mixed by inversion and allowed to remain at 4 °C for at least 12 h. RNase A (50 µg µl^{-1}) was added to the supernatant and allowed to incubate for 60 min at 37 °C. The supernatant was phenol:chloroform extracted (1:1, v/v), ethanol precipitated overnight, and resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The DNA (2–5 µg) was electrophoresed in 3% agarose and visualized with ethidium bromide.

DNA fragmentation assay using Hoescht fluorescence [15, 16]

Cells (10^6) were lysed in 0.1% Triton X-100, 5 mM Tris, 20 mM EDTA, pH 8.0. After thorough mixing, the lysate was centrifuged at 30 000 × **g** at 4 °C for 40 min. Aliquots (50 µl) were added to 2 ml of TNE (100 mM Tris, 10 mM EDTA, 2 M NaCl, pH 7.4) and 0.1 ng ml^{-1} Hoechst 33258 dye (Sigma). DNA lost to the media could be measured in the same way by first adjusting 1 ml of media to 25 mM EDTA and centrifuging at 20 000 × **g** at 4 °C for 40 min. A 200 µl aliquot of the supernatant was added to 2 ml TNE and Hoechst 33258 for fluorescence measurement. Fluorescence was measured in a luminescent spectrophotometer at excitation wavelength of 365 nm and emission wavelength of 460 nm. DNA values were calculated by comparison with a standard curve of calf thymus DNA.

LIPID ANALYSES

Quantitation of lipid formation by [^{3}H]palmitic acid labelling

In order to study sphingomyelin catabolism, cells were cultured for 24 h in media containing 10 µCi [^{3}H]palmitate (equilibrium labelling), appropriate drugs were added,

cells harvested, washed and lipids extracted as described previously [17]. Briefly, harvested cells (10^6) were washed twice with ice-cold PBS then taken up in 1 ml methanolic HCl (1%). After vortexing, 2 ml chloroform and 0.6 ml distilled-deionized water were added with sonication and vortexing. The two phases were separated by centrifugation at 1000 × **g** for 5 min, and the upper, aqueous phase removed. The total lipid fraction recovered from the lower phase was analysed by high-performance thin-layer chromatography (HPTLC). The [^{3}H]ceramide formed was resolved from cholesterol, free fatty acids and diacylglycerols by HPTLC in chloroform:methanol:acetic acid:water (85:4.4:5:0.5 v/v/v/v), sprayed with En^{3}Hance to facilitate autoradiography and the bands scraped for radioactivity determination by liquid scintillation counting.

Quantitation of ceramide levels by diacylglycerol kinase assay

A modification of the method of Preiss *et al.* [18] was used to determine ceramide mass. Lipids were isolated from 10^6 cells by the extraction method described above. The lower organic phase was dried down in a Speed Vac, redissolved in 0.5 ml of chloroform:methanol (2:1) and transferred to a clean screw top microfuge tube and again dried down. The enriched lipid fraction was dissolved in 20 µl of a 7.5% n-octyl-β-glucopyranoside, 5 mM cardiolipin, 1 mM DETAPAC solution by heating to 70 °C, followed by sonication and vortexing. The volume was then brought to 100 µl containing 72 mM imidazole, 12.5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM EGTA, 0.4 mM DETAPAC, 50 mM LiCl, 50 µg ml^{-1} DAG kinase and 1 mM ATP (5 µCi). After 30 min at room temperature, phosphorylated lipids were extracted in the same manner as above except that 1% perchloric acid was used in place of DDI water. The samples were spotted on HPTLC plates and developed in chloroform:acetone: methanol:acetic acid:water (10:4:3:2:1) or chloroform: methanol:acetic acid (65:15:5). Authentic standards of ceramide-1-phosphate (synthesized in the same manner except pure ceramide and cold ATP is used) or phosphatidic acid can be used for identification of bands. The bands were scraped into scintillation vials and counted either with or without scintillation fluid (Cherenkov counting).

Results

Effect of staurosporine on neurotumour cell viability

In order to develop a model to study programmed cell death in neurotumour cells, the effect of staurosporine as an apoptosis inducing agent was first assessed by determining to what extent neurotumour cells could be killed by staurosporine. HN-2 cells (immortalized hippocampal neurons) were treated with increasing concentrations of staurosporine and their viability determined by the MTT assay, which measures mitochondrial function, and Coulter counting, which counts intact nuclei which have been released from cell bodies. Figure 1 shows that as little as 50 nM staurosporine is capable of killing from 75 to 90% of the cells treated in a 24 h time period when measured by the MTT assay or the cell nuclei counting method. From the kinetics of PCD it would appear that the mitochondria are slightly more affected by treatment with staurosporine than dissolution of the nucleus. However, there is good correlation between the two methods of determining cellular viability.

Effect of C2-ceramide on neurotumour cell viability

HN-2 cells were treated with increasing concentrations of C2-ceramide (*N*-acetylsphingosine) and their viability was measured by MTT and Coulter counting. Figure 2 shows that increasing concentrations of C2-ceramide kill HN-2 cells in a dose-dependent manner. A dose of 25 µM C2-ceramide kills between 75 and 85% of the cells in 24 h. Again we see the same pattern of mitochondrial function being more rapidly affected than the nucleus.

Staurosporine and ceramide induce DNA damage

The potential capacity of staurosporine, ceramide, and inducers of ceramide formation to promote DNA fragmentation characteristic of programmed cell death was assessed. Neurotumour cells were treated with ceramide, staurosporine for 24 h, the DNA isolated, resolved on a 3% agarose gel, and visualized with ethidium bromide. The ceramidase inhibitor, oleoylethanolamine [19] and

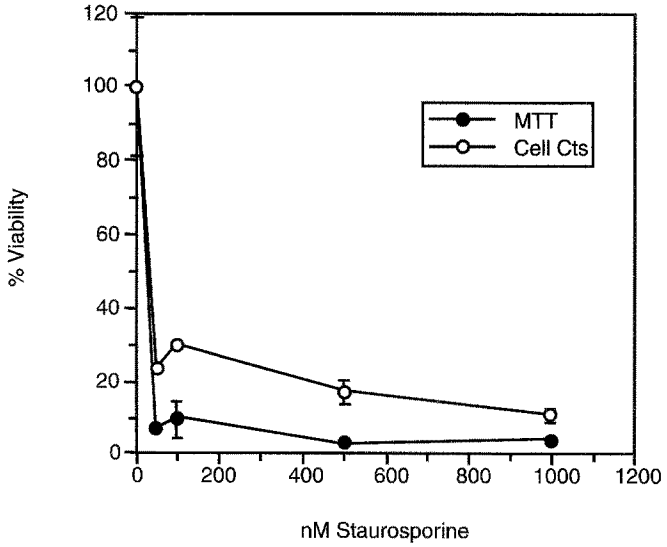

Figure 1. Effect of staurosporine on cellular viability. HN-2 cells in serum free media were treated with the indicated doses of staurosporine in DMSO for 24 h. Cellular viability was determined by Coulter counting and MTT assay. Error bars represent SD of three experiments.

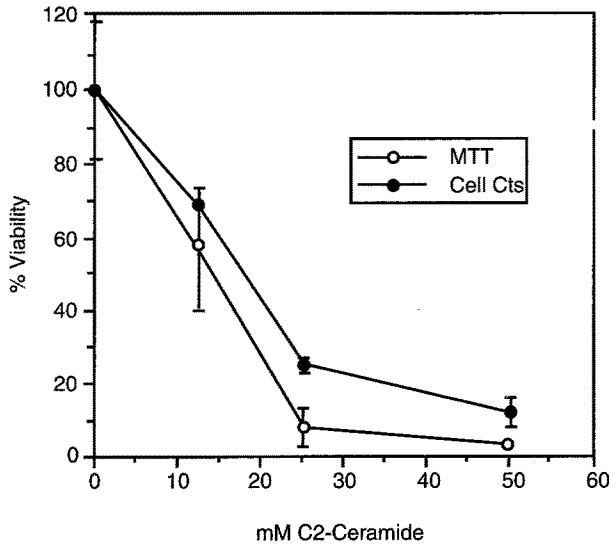

Figure 2. Effect of C2-ceramide on cellular viability. HN-2 cells in serum free media were treated with the indicated doses of C2-ceramide in DMSO for 24 h. Cellular viability was determined by Coulter counting and MTT assay. Error bars represent SD of three experiments.

bacterial sphingomyelinase, which are capable of increasing ceramide formation *in vivo* were also examined. Figure 3 shows that ceramide and staurosporine are capable of inducing the DNA to fragment into oligonucleosomal fragments that, when run on agarose gels, form the typical ladder pattern. Agents that induce the formation of endogenous ceramide, oleoylethanolamine and bacterial sphingomyelinase were also able to produce the DNA laddering pattern. The observation that C2-ceramide and inducers of ceramide formation can cause PCD suggests that ceramide may indeed be an important participant in the PCD signalling cascade.

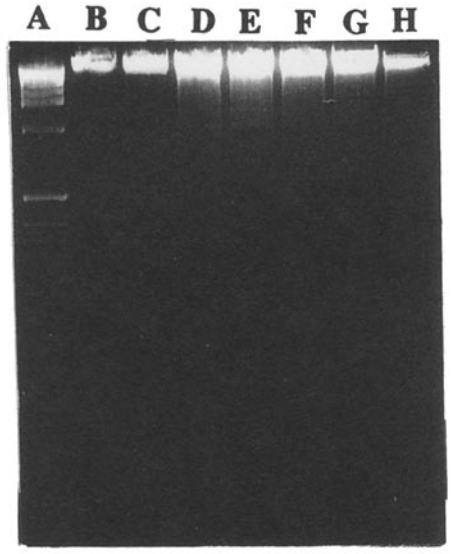

Figure 3. Staurosporine and ceramide induce DNA fragmentation and laddering. HN-2 cells (immortalized hippocampal cells) were treated in serum free media for 24 h, their DNA isolated and resolved on a 3% agarose gel. Lane A, DNA Standard; B, vehicle (DMSO); C, 20 mM C2-ceramide; D, 40 μM C2-ceramide; E, 500 nM staurosporine; F, 500 nM staurosporine and 50 μM oleoylethanolamine; G, 50 μM oleoylethanolamine; H, 1 U ml^{-1} sphingomyelinase.

Staurosporine causes DNA to fragment into the cytosol

The degradation of DNA into oligonucleosomal fragments that are characteristically associated with advanced stages of apoptosis can be measured fluorometrically and quantitated. Exposure of F-11 cells to increasing concentrations of staurosporine resulted in increasing levels of fluorescence due to increased fragmentation of DNA (Fig. 4). Exposure to 600 nM staurosporine for 24 h induced a three-fold increase in DNA fragmentation fluorescence over that of basal levels. Higher doses of staurosporine resulted in decreasing fluorescence found in the cytosol and a concomitant increase in fluorescence found in the media (Data not shown). The likely reason is that the cells begin to lose membrane integrity and the DNA fragments leak in to the surrounding media.

Staurosporine induces ceramide formation

The effect of staurosporine on sphingomyelin/ceramide metabolism was assayed by pulsing the cell with [^{3}H]palmitate for 24 h then treatment with staurosporine and analysis of [^{3}H] incorporation into lipids. We found that treatment with 300 nM staurosporine caused a 100%

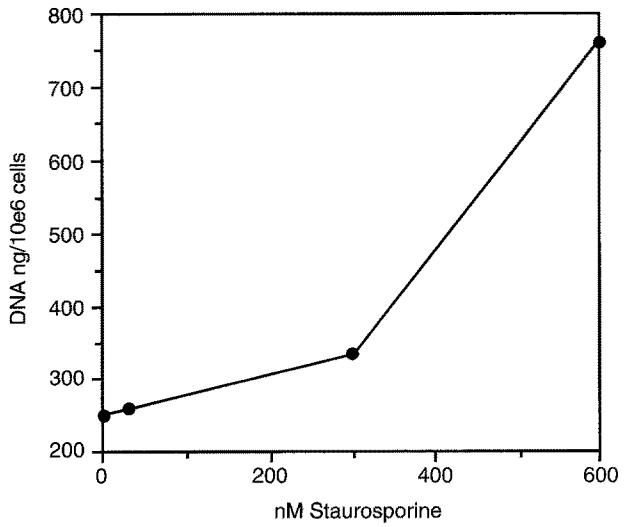

Figure 4. Staurosporine induces DNA fragmentation into the cytosol. F-11 cells (transformed dorsal root ganglion) were treated in serum free media with indicated doses of staurosporine for 24 h. The cytosolic fraction (supernatant of a 40 k × **g** spin) was assayed for DNA fragmentation using Hoechst 33258 dye. A DNA standard curve was determined using calf thymus DNA. Result are representative of two separate experiments.

increase in the formation of labelled ceramide. In addition, we observed a 20% decrease in the amount of sphingomyelin labelled (Fig. 5) but no changes in the glycosphingolipids (data not shown). When one considers that there is approximately a five-fold difference in the mass of sphingomyelin compared to ceramide found in cells, the increase in ceramide becomes stoichiometrically similar to the decrease in sphingomyelin. This, when considered with previous work showing that Fumonisin B1 (which inhibits sphingolipid synthesis *de novo*) [20] does not block PCD caused by inducers of ceramide formation or the activation of sphingomyelinases (unpublished results), it is reasonable to assume that the source of ceramide is the catabolic degradation of sphingomyelin. Furthermore, when the total mass of ceramide is measured, we find approximately a four fold increase in ceramide content in cells treated with staurosporine for 24 h (Fig. 6).

Oleoylethanolamine induces ceramide formation and PCD

Oleoylethanolamine is an inhibitor of ceramidase, the enzyme responsible for the catabolism of ceramide to sphingosine and fatty acid. We observed previously (data not shown) and in this report that treatment of cells with OE is sufficient to cause apoptosis (Fig. 3). The most likely mechanism is through the inhibition of ceramidase [21]. By blocking the catabolism of ceramide, the result is increased cellular ceramide levels. We have found that treatment of cells with oleoylethanolamine by itself causes a modest increase in ceramide levels (data not shown) and that concomitant treatment of the cells with staurosporine and oleoylethanolamine, greatly increases the level of ceramide formation (Figs 5 and 6) over that of staurosporine alone. Furthermore, we can also show that the addition of oleoylethanolamine in conjunction with staurosporine will increase the potency of staurosporine as an inducer of PCD (Fig. 7).

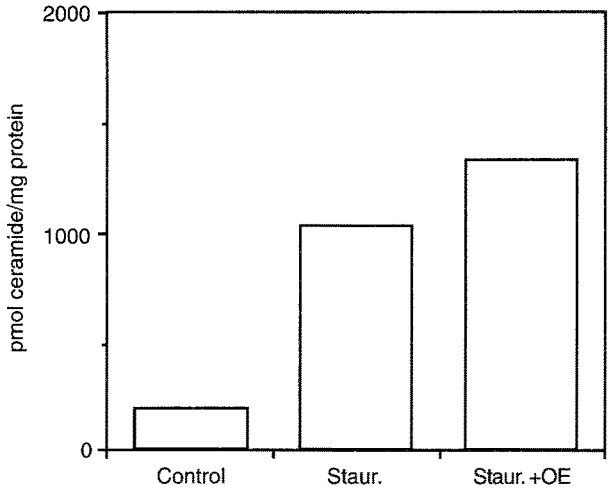

Figure 6. Effect of staurosporine and oleoylethanolamine on total ceramide mass. HN-2 cells were treated with 200 nM staurosporine with and without 25 μM oleoylethanolamine for 24 h in serum free media. Following treatment, the lipids were isolated and subjected to [^{32}P]ATP phosphorylation by DAG kinase. Ceramide-1-^{32}P was isolated by HPTLC and quantitated by scintillation counting. The results represent the average of two separate experiments.

Discussion

In this report we explore the role of ceramide as an important part of the signalling mechanism to initiate the

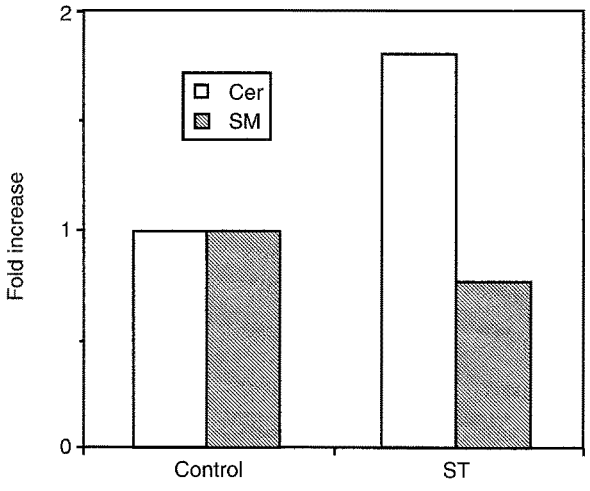

Figure 5. Effect of staurosporine on sphingomyelin-ceramide metabolism. HN-2 cells were pulsed with [^{3}H]palmitate for 24 h then treated with 500 nM staurosporine for 24 h in serum free media. The lipids were isolated analysed by HPTLC, autoradiography and scintillation counting (see text for experimental details). Results are representative of two separate experiments.

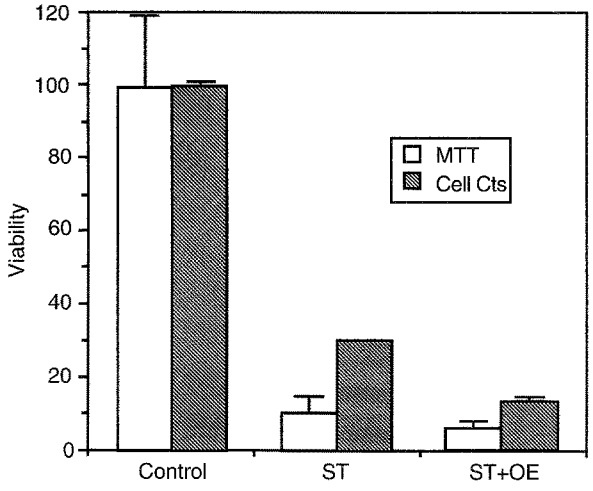

Figure 7. Effect of staurosporine and oleoylethanolamine on cellular viability. HN-2 cells were treated with 200 nM staurosporine with and without 25 μM oleoylethanolamine for 24 h in serum free media. Following treatment, cellular viability was determined by Coulter counting and MTT assay. Error bars represent SD of three separate experiments.

cell death programme. We have shown that staurosporine, a broad based inhibitor of protein kinase C, is a potent inducer of PCD in neurotumour cells. Our data showing that ceramide also is capable of inducing PCD and that staurosporine causes increases in ceramide formation suggest that the formation of ceramide is a possible mechanism of the action of staurosporine. This idea is supported by the ability of the ceramidase inhibitor, oleoylethanolamine, to potentiate the action of staurosporine both in terms of ceramide production and cell killing. Acute treatment of several cell types with protein kinase C inducers, such as phorbol esters or diglycerides, have been claimed to block cytokine or calcium induced PCD [22–24]; however, this protection is lost with chronic treatment [25, 26]. This suggests that protein kinase C has a cell-protective activity and its inactivation may result in sphingomyelinase activation, ceramide formation and programmed cell death.

We have previously shown that ceramides (but not dihydroceramides or glycosylated ceramides) induce apoptosis (DNA laddering and cell death after 24 h) in immature B-cells (WEHI-231) [5, 27] and that a physiological paradigm (addition of anti-IgM to induce cross-linking of surface IgM molecules and PCD) leads to a two-fold increase in ceramide mass and four-fold increase in both the ceramide:cholesterol and the ceramide:-sphingomyelin ratio over a 24 h period [5]. We have also shown that staurosporine can induce PCD in chick neurons and astrocytes through the generation of ceramide (unpublished data). Interestingly our observed slow release of ceramide differs from the reported ability of cytokines such as tumour necrosis factor α and interleukin-1b to rapidly (<10 min) form ceramide from sphingomyelin in various cell lines of haematopoietic origin (HL-60, U-937, etc.) [4, 10, 28–30] and then cause apoptosis over the next several hours. We did not see any rapid changes in ceramide levels either in WEHI cells or in astrocytes, neurons or neuronally-derived cells. Further, we need the continued presence of ceramide or anti-IgM to induce PCD since the process of PCD is reversible for up to 6–10 h [31] we therefore believe that the slow, sustained release of ceramide is physiologically significant.

Apoptosis or PCD is generally believed to perform a beneficial function in the nervous system by removal of dysfunctional elements without inflammation [32]. PCD is of particular interest in neurons because of the huge die-off of neurons during embryonic development and apoptosis has a potential role in neurodegenerative diseases such as Alzheimer. Thus the β-amyloid (AbP) peptide [32] may act to induce apoptosis and increase the sensitivity of the neuron to growth factor deprivation, ischaemia, excitatory amino acids, stress, etc., resulting in progressive neurodegeneration and loss of cognitive function. Although ceramide has been shown to induce apoptosis in several cell types it has not previously been shown to induce cell death by apoptosis in neurons. The effects of exogenous C-2 ceramide and agents which increase both cellular ceramide levels and apoptosis (staurosporine), appear similar in both embryonic neuron primary cultures and in neurotumour cell lines. This suggests that the activation of membrane-bound sphingomyelinase and formation of ceramide, over a period of 24 h, is a key event in neuronal apoptosis and that a cascade of ceramide-activated kinases and phosphatases, leading to endonuclease action and DNA fragmentation will be identified in neurons.

Brain cancer is notorious for being resistant to all types of treatment. This study and others give evidence of the role of ceramide as an inducer of PCD as well as the importance of the sphingomyelin cycle in transducing the apoptosis signal. Further research may determine that the sphingomyelin cascade is similar in importance as the phosphatidylinositol cascade for signal transduction in that both generate a membrane-anchored lipid (ceramide and diacylglycerol, respectively) which can recruit to the membrane and thereby activate cytosolic kinases. The importance of determining the nature of the apoptotic signal is crucial to designing approaches that take advantage of this natural method of removing hazardous cells, and thus protecting the whole organism. This work shows the ability of staurosporine, ceramide and agents that produce ceramide can cause PCD in neurotumour cells. It is possible that this may lead to approaches which take advantage of the sphingomyelin-ceramide pathway, causing an increase in ceramide levels through agents such as staurosporine and oleoylethanolamine and assist the organism's own anticancer response of causing affected cells to die.

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