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Suramin induced ceramide accumulation leads to apoptotic cell death in dorsal root ganglion neurons

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Abstract

Suramin is an experimental antineoplastic agent that is currently being tested in clinical trials for a number of human cancers. In previous clinical trials, it has been noted that a significant percentage of patients treated with suramin develop a peripheral neuropathy. Both the cytotoxic (chemotherapeutic) and neurotoxic mechanisms of action of this compound are unknown. Evidence presented in this study suggests that both effects may be due to extensive disruption in glycolipid transport and/or metabolism. Suramin treated dorsal root ganglion cultures revealed an accumulation of the GM_1 ganglioside and ceramide. Exposure of cultures to suramin, a cell permeable ceramide analog, or sphingomyelinase lead to apoptotic cell death demonstrated by electron microscopy, *bis*-benzimide staining and DNA laddering on gel electrophoresis. Furthermore, a significant increase in intracellular ceramide preceded cell death in suramin treated neurons. We propose that suramin induced ceramide accumulation within neurons leads to apoptotic cell death.

Keywords: apoptosis; ceramide; peripheral neuropathy; chemotherapy; dorsal root ganglion

Abbreviations: DRG, dorsal root ganglion; LIB, lamellar inclusion bodies; NGF, nerve growth factor; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; MS, mass spectrometry

Introduction

Suramin is a polysulfonated naphthylurea developed subsequent to observations on the trypanocidal activity of the azo dyes trypan red and trypan blue (Hawking, 1978). It has recently been used as an experimental chemotherapeutic agent for the treatment of a number of human malignancies (Broder *et al*, 1985; Stein *et al*, 1988; La Rocca *et al*, 1990a; Pollak and Richard, 1990; LaRocca *et al*, 1991). Initial clinical trials revealed major toxicities.

In one study, 80% of patients experienced symptoms of peripheral neuropathy (LaRocca *et al*, 1989). A subsequent study reported that in patients with disseminated cancer given suramin; four developed a sensorimotor neuropathy with electrophysiologic and histologic features of axonal degeneration and segmental demyelination (LaRocca *et al*, 1990b). Neuropathy was most likely to occur in those patients with peak plasma suramin levels above 350 μg/ml, implying a dose response effect. Thus, severe peripheral neuropathy is a major dose limiting effect of suramin.

In spite of major toxicities, suramin has been documented to have significant *in vitro* and *in vivo* activity against a variety of solid tumor cell lines at drug concentrations that are clinically achievable (Betscholtz *et al*, 1986; Coffey *et al*, 1987; Kim *et al*, 1991; Gansler *et al*, 1992). For this reason, there continues to be considerable clinical interest in this drug. However, the mechanism of anti-tumor activity and neurotoxicity of the drug remain unknown. Most reports on suramin describe an inhibitory effect on growth factor receptor binding as its cytotoxic mechanism of action (Hosang, 1985; Coffey *et al*, 1987; Sjölund and Thyberg, 1989; Pollack and Richard, 1990). Other studies have reported a stimulatory effect by suramin on growth factor receptors (Mahoney *et al*, 1990; Cardinali *et al*, 1992; Sartor *et al*, 1992; Tsutsumi *et al*, 1993; Tsutsumi *et al*, 1994). This suggests a pleiotropic effect of suramin on growth factor receptors. Previous studies from our laboratory indicate that suramin's interaction with the high affinity nerve growth factor (NGF) receptor (TrkA) probably does not play a role in drug induced neurotoxicity (Gill *et al*, 1996; Gill and Windebank, 1998).

Other studies have suggested that suramin treatment (*in vitro* and *in vivo*) leads to the accumulation of lamellar inclusion bodies (LIB) that morphologically resemble those observed in lysosomal storage diseases (Buys *et al*, 1978; Rees, 1978; Constantopoulos *et al*, 1981; Rees *et al*, 1982; Akanji, 1988). In dorsal root ganglion (DRG) neurons *in vitro* and *in vivo*, characteristic LIB begin to form within hours of suramin exposure; dying cells are full of LIB (Gill *et al*, 1995). There is a concomitant rise in intracellular GM_1 ganglioside levels localized to LIB.

A key element in this study is investigating ways to separate the neurotoxic effects of chemotherapeutic agents from their cytotoxic efficacy. In particular, we wish to understand why an anti-cancer drug designed to kill rapidly dividing cells would injure the DRG, composed of post-mitotic primary afferent sensory neurons and post-mitotic Schwann cells involved in myelination. A potential link between rapidly dividing cancer cells and post-mitotic DRG neurons is their relatively high rates of glycosphingolipid (ganglioside) synthesis.

From our work and others, it is clear that suramin treatment results in glycolipid accumulation. The focus of this study was to characterize the mechanism of neuronal

(DRG) cell injury and cell death associated with sphingolipid defects. Such studies propose an alternative cytocidal mechanism mediated by suramin, namely the accumulation of ceramide, and its role in programmed/apoptotic cell death. This work contributes to established findings describing the role of sphingolipids in signal transduction and cell regulation as a mechanism of growth suppression and cell death (Hannun and Linardic, 1993; Obeid *et al*, 1993; Hannun and Obeid, 1995; Brugg *et al*, 1996).

Results

Cell viability studies revealed a dose dependent reduction in neuron survival in suramin treated cultures (Figure 1). Cultures exposed to 300 and 600 μM suramin revealed a significant reduction in neuron viability after 48 h of treatment (81%$\pm$4.1 and 75%$\pm$3.9 cell viability, respectively). Neuronal loss continued with length of suramin exposure: 40% ($\pm$4.9) and 16% ($\pm$5.2) cell viability after 10 days exposure to 300 and 600 μM suramin, respectively. In subsequent studies, DRG cultures were exposed to 300 μM suramin for 12 h to 4 days. This concentration of suramin is comparable to doses of drug reported in clinical and animal studies to precipitate a peripheral sensory neuropathy.

Lysosomal accumulation of GM_1 ganglioside

Electron microscopic studies revealed dying neurons full of LIB (Figure 2). Suramin treated cultures revealed apoptotic neurons with characteristic nuclear fragmentation and intact nuclear and cytoplasmic membranes. DRG cultures labeled with Texas Red-dextran displayed an abundance of lysosomes/inclusion bodies within the cytoplasm of suramin treated DRG neurons (Figure 3). Limited fluorescent lysosomal profiles were seen in untreated cultures. Suramin treated DRG cultures accumulated monosialoganglioside GM_1 as detected by cholera toxin-β subunit labeling (Figure 4A). Positive and punctate staining was seen within the cytoplasm and plasma membrane. GM_1 labeling was not observed within the nucleus. The punctate ganglioside staining revealed a cellular distribution similar to that observed in cultures labeled with Texas Red-dextran. Cultures exposed to suramin and the glucosylceramide synthase inhibitor PDMP revealed reduced levels of GM_1 ganglioside via cholera toxin-β staining. Inhibition of ganglioside biosynthesis with exposure of cultures to PDMP did not prevent suramin induced cell death (Figure 4B). DRG cultures

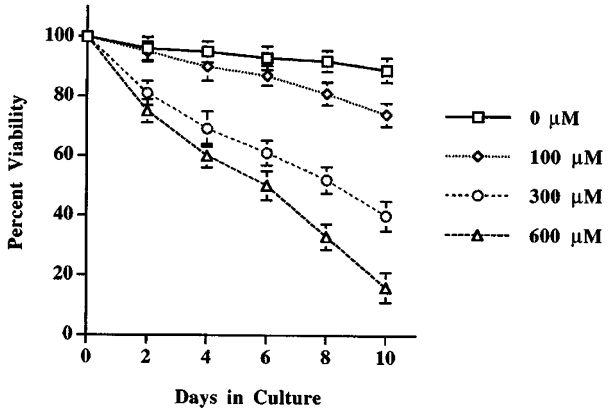

Figure 1 Neuron survival was studied using phase contrast microscopy and grid counting. The number of viable neurons was determined after exposure to 100, 300, and 600 μM suramin. Neuron-enriched DRG cultures exposed to either 300 or 600 μM suramin revealed a significant reduction in cell viability after 2 days in culture ($P<0.01$ Student's *t*-test). DRG cultures revealed a dose dependent reduction in neuronal viability with continued exposure to suramin. Cultures exposed to subtoxic concentrations of suramin (100 μM) only revealed a significant reduction in neuron viability after 8 days in culture

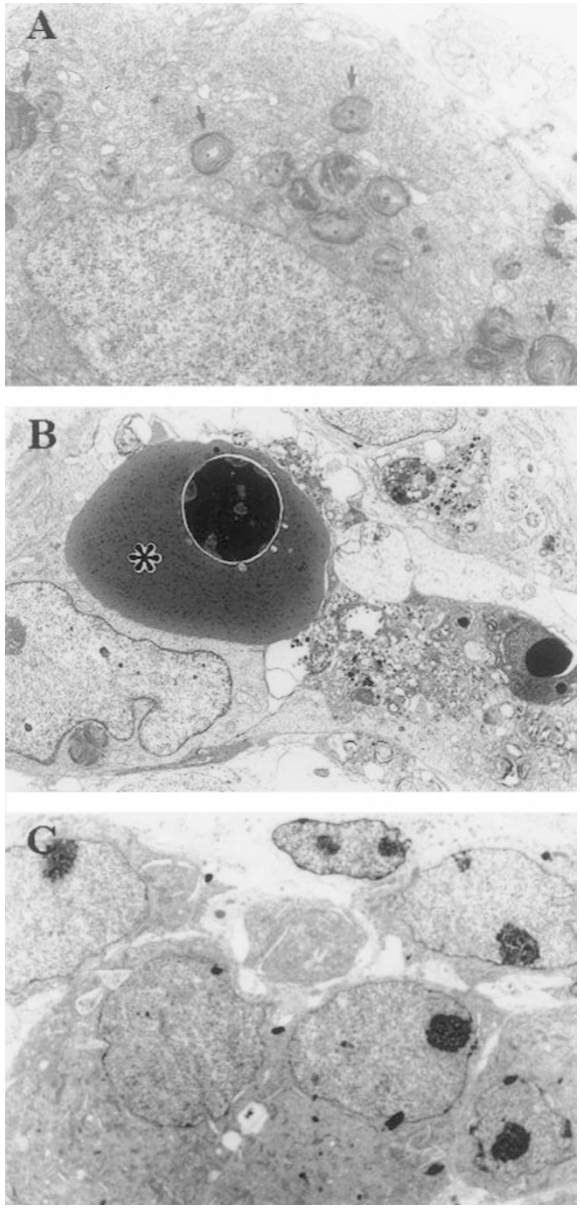

Figure 2 Electron micrographs from rat DRG cultures treated with 300 μM suramin for 24 h revealed the accumulation of LIB (**A**; arrow, mag $\times$ 4800). Suramin treatment for 48 h (**B**; mag $\times$ 2800) revealed changes consistent with apoptosis in a neuron (*) displaying condensed chromatin and intact cell and nuclear membranes. Control cultures (**C**; mag $\times$ 2100) did not display LIB and apoptotic figures were rarely seen

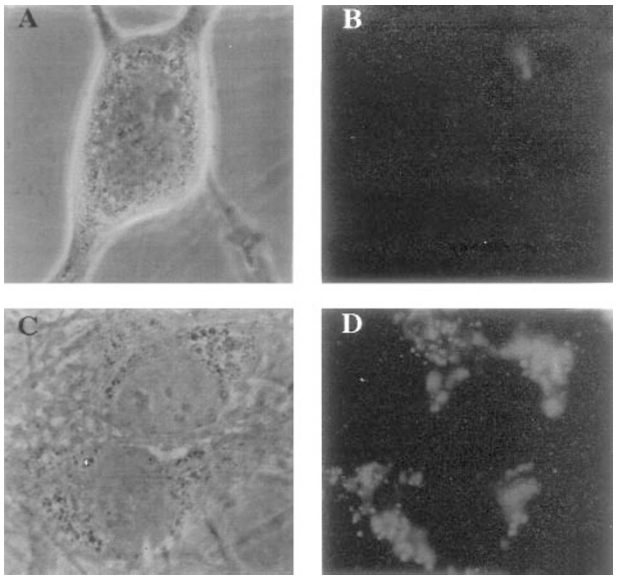

Figure 3 DRG neuronal cultures went untreated (**A,B**) or exposed to 300 μM suramin (**C,D**) for 48 h. Under phase contrast microscopy, suramin treated neurons (**C**) have an abundance of inclusion bodies which are detected as lysosomal profiles with Texas Red-dextran under fluorescence microscopy (**D**). Untreated neurons did not reveal any significant LIB under phase (**A**) or fluorescence micrsocopy (**B**; mag $\times$ 400)

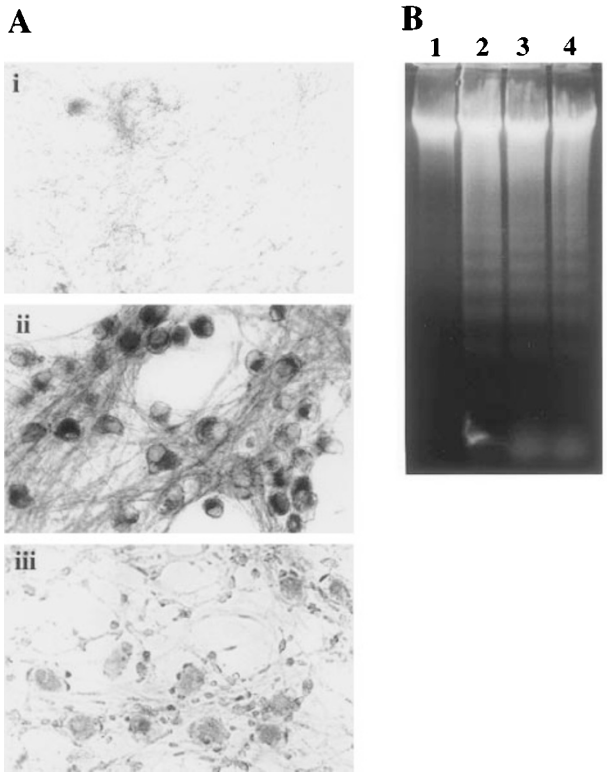

Figure 4 (**A**) DRG cultures were treated with 300 μM suramin for 48 h, fixed, and immunostained with cholera toxin-β subunit, a specific marker for GM_1 ganglioside. Positive staining was observed with the brown chromagen diaminobenzidine. Suramin treated cultures (ii) revealed an accumulation of GM_1 ganglioside within the cytoplasm of neurons and along neuroitic processes as compared to untreated cultures (i). The punctate cholera toxin-β staining of GM_1 ganglioside within the cytoplasm of neurons co-distributed with LIB/lysosomal staining via Texas Red-dextran labeling in Figure 3. Cultures exposed to suramin and 10 μM of *d,l-threo*-PDMP (iii); (an inhibitor of glucosyl ceramide synthase) reduced the level of GM_1 ganglioside staining. Neuronal cultures exposed to suramin and the inactive diastereomer *d,l-erythro*-PDMP revealed GM_1 ganglioside accumulation comparable to cultures exposed to suramin alone (data not shown). (**B**) Neuron-enriched DRG cultures were exposed to 300 μM suramin alone (lane 2) or with the addition of 10dμM *d,l,threo*-PDMP (lane 3) or 10 μM *d,l-erythro*-PDMP (lane 4) for 48 h. Internucleosomal fragmentation by gel electrophoresis, characteristic of apoptotic cell death, was observed in cultures exposed to suramin alone as well as those exposed to suramin and PDMP. Untreated/control cultures (lane 1) revealed intact genomic DNA

exposed to 300 μM suramin, with or without 10 μM PDMP, revealed internucleosomal fragmentation by gel electrophoresis indicative of apoptotic cell death.

Suramin induced apoptosis in DRG neurons

Apoptotic changes were observed in suramin treated DRG cultures by electron microscopy (Figure 2) and *bis*-benzimide stain (Figure 5). Under fluorescence microscopy, neuronal nuclear and cellular condensation and fragmentation were observed by 36 to 48 h of suramin treatment (Figure 5). Phase contrast images revealed intact cellular membranes. Condensed and fragmented nuclei observed with *bis*-benzimide stain were only observed in Schwann cells and fibroblasts of DRG cultures exposed to suramin for longer than 72 h. Nuclear changes characteristic of apoptosis were also observed by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique (data not shown) (Pittman *et al*, 1993; Piqueras *et al*, 1996). Enriched DRG neuronal cultures were established and used to study the integrity of genomic DNA after suramin treatment (Figures 4 and 6). DNA laddering suggestive of internucleosomal strand digestion was observed with suramin treatment (300 μM for 48 h), correlating with morphological evidence indicative of apoptosis. In all of the above methods used to study apoptotic death, suramin (300 μM) induced neuronal apoptosis was observed by 36–48 h treatment *in vitro*.

Ceramide mediated apoptosis in DRG neurons

DRG cultures exposed to 10 μM of the short chain ceramide analog C_2-ceramide and 300 mU/ml sphingomyelinase were also stained with *bis*-benzimide and TUNEL. *Bis*-benzimide staining revealed apoptotic profiles in DRG neurons exposed to sphingomyelinase and C_2-ceramide similar to those in suramin treated cultures (Figure 5). Apoptotic cells, as determined by nuclear condensation and blebbing were first observed at 24 h exposure to both C_2-ceramide and sphingomyelinase. TUNEL analysis also revealed nuclear fragmentation in neurons exposed to C_2-ceramide (data not shown). As a positive control for neuronal apoptosis, DRG neurons were cultured in the absence of NGF, which revealed apoptotic profiles by 12–24 h. This model of neuronal apoptosis has been described previously (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994).

Enriched DRG neuronal cultures treated with C_2-ceramide also revealed DNA laddering by gel electrophor-

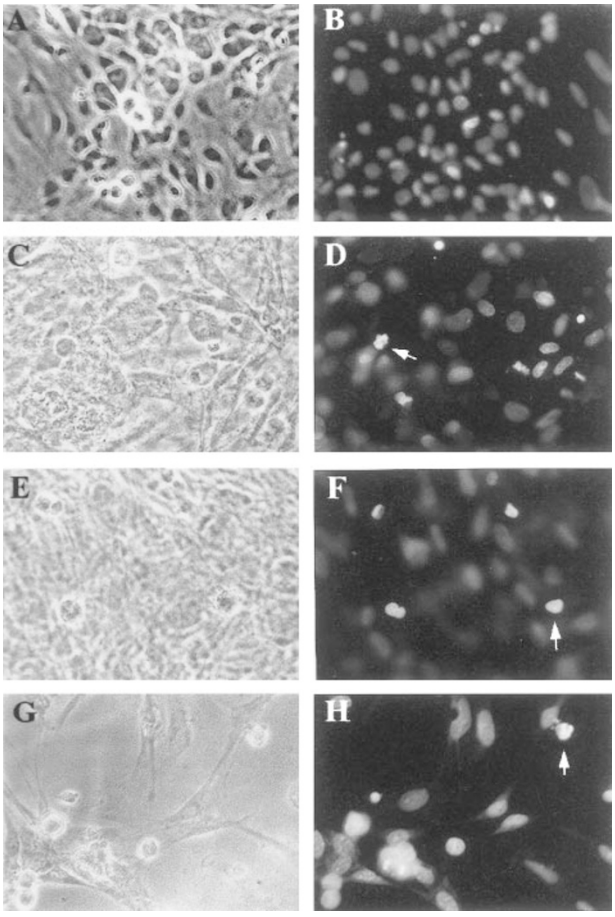

Figure 5 DRG cultures were exposed to 300 μM suramin (**C,D**; 48 h), 10 μM C_2-ceramide (**E,F**; 24 h) or 300 mU/ml sphingomyelinase (**G,H**; 24 h), fixed and stained with *bis*-benzimide (Hoechst 33258). Phase (**A,C,E,G**) and fluorescence (**B,D,F,H**) microscopic images of representative areas were obtained (mag $\times$ 100). Apoptotic cells (arrow) identified with condensed and fragmented nuclei with *bis*-benzimide stain were observed in cultures treated with suramin, C_2-ceramide, and sphingomyelinase. Similar apoptotic bodies were not observed in untreated cultures (**A,B**)

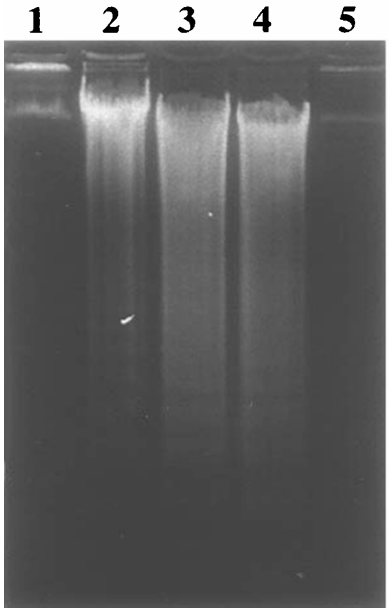

Figure 6 Neuron-enriched DRG cultures were exposed to NGF withdrawal (lane 2), 300 μM suramin (lane 3), 10 μM C_2-ceramide (lane 4), or 10 μM dihydroceramide (lane 5) for 48 h. Isolated DNA was separated on a 1.2% agarose gel and visualized by ethidium bromide. DNA laddering characteristic of apoptosis was observed in cultures exposed to suramin and C_2-ceramide. NGF withdrawal was used as an established model of neuronal apoptosis and DNA laddering. Untreated cultures and those exposed to dihydroceramide (an inactive C_2-ceramide analog) did not induce DNA laddering (lanes 1 and 5, respectively)

esis (Figure 6). Similar profiles of internucleosomal fragmentation manifested as DNA laddering were observed in cultures treated with suramin and those cultured in the absence of NGF. Untreated cultures and those exposed to 10 μM dihydroceramide for 48 h did not reveal any DNA laddering.

Ceramide measurement

Enriched neuronal cultures were used to investigate changes in total intracellular ceramide levels induced by 300 μM suramin exposure. Two independent means were used to quantitate changes in intracellular ceramide: the diacyglycerol kinase assay and MS analysis. Accumulation of total intracellular ceramide was observed by the diacylglycerol kinase assay (Figure 7A). These studies revealed a 2.53 fold increase over untreated neuronal cultures of intracellular ceramide after 24 h of suramin (300 μM) treatment and 5.73 fold increase in cultures exposed to drug for 48 h. Electrospray ionization MS analysis was performed as described in a recent report (Figure 7B) (Watts *et al*, 1997). Total ceramide levels in neuron enriched DRG cultures increased significantly by 12 h exposure to 300 and 600 μM suramin (132% $\pm$ 8.5 and 144% $\pm$ 11.0 of control, respectively). Ceramide levels increased further after 24 h suramin treatment. Cultures exposed to subtoxic concentrations of suramin (100 μM) did not product significant changes in ceramide levels as compared to untreated cultures.

Discussion

In the present study, sensory neurons exposed to the chemotherapeutic agent suramin displayed distinct structural and biochemical changes compatible with apoptotic cell death. Electron microscopic analysis of suramin treated DRG neurons revealed characteristic apoptotic changes; condensed cells, nuclear condensation and fragmentation, and intact cell and nuclear membranes. *In vivo* studies of suramin treated rats have revealed similar profiles of apoptotic cell death in DRG neurons (Russell and Windebank, 1993). Additional evidence of suramin induced apoptosis was revealed by *bis*-benzimide staining and internucleosomal fragmentation with gel electrophoresis. Evidence of necrotic swelling was observed in suramin treated cultures only at prolonged exposure times ($>$72 h). This is consistent with other reports of necrotic cell death following apoptotic injury (Wyllie *et al*, 1980; Wyllie, 1985).

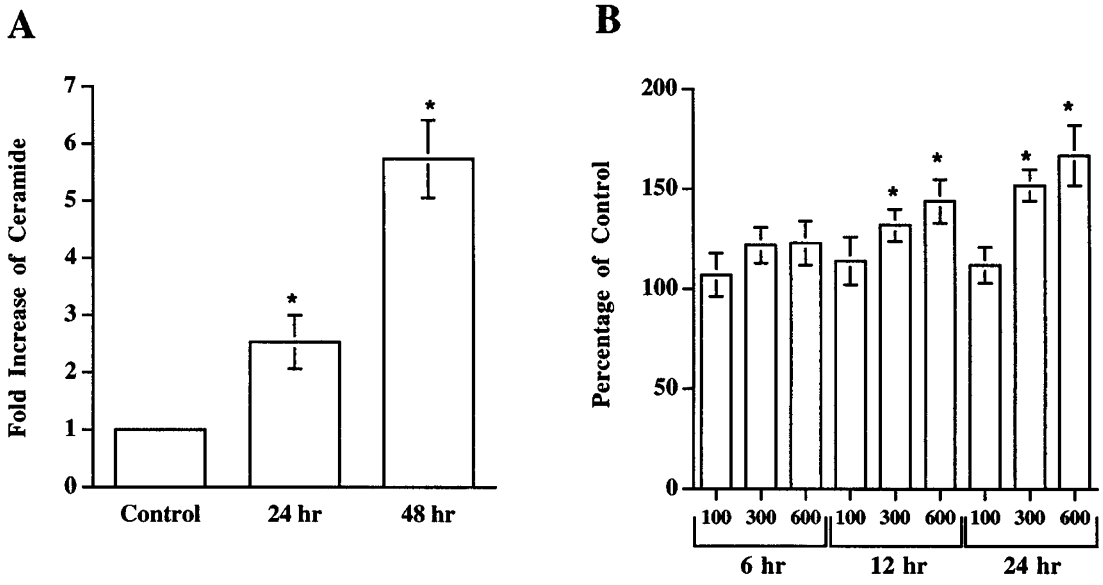

Figure 7 (**A**) Intracellular ceramide was measured by the diacylglycerol kinase assay from neuron-enriched DRG cultures. Densitometry revealed that 300 μM suramin treatment resulted in a 2.53 ($\pm$0.47) and 5.74 ($\pm$0.68) fold increase in intracellular ceramide at 24 and 48 h, respectively. Ceramide measurements were the mean of three experiments (error bars represent S.E.M.; *indicates a significant change as compared to untreated cultures, $P<0.01$ Student's *t*-test). (**B**) Cellular sphingosine-based ceramide levels in suramin treated neuron-enriched DRG cultures were also determined by MS analysis. Cultures were exposed to 100, 300 or 600 μM suramin for 6, 12 or 24 h. Lipid extractions and MS analyses were performed as described in Materials and methods. Signal intensitities of all ceramide families (summation of peaks from 500 to 990 m/z) were normalized to signal intensity from internal C2 : 0 standard. Values plotted are relative signal intensities as percentage of control/untreated cultures. Cultures exposed to 300 and 600 μM suramin revealed significant changes in total ceramide levels after 12 and 24 h exposure (*indicates a significant change as compared to untreated cultures, $P<0.01$ Student's *t*-test). MS analyses were the mean of three experiments (error bars represent S.E.M.)

The monosialoganglioside GM_1 is the predominant glycosphingolipid in developing DRG neurons. The significance of drug induced GM_1 ganglioside accumulation regarding suramin mediated neurotoxicity was clearly warranted. In our study, inhibition of endogenous ganglioside biosynthesis in suramin treated neuronal cultures reduced the levels of cholera toxin-β staining of GM_1 ganglioside but was not able to rescue the cells from drug induced apoptosis. While these findings suggested that ganglioside accumulation by itself is not sufficient to promote suramin induced neuronal damage, it allowed us to focus on the effects of glycosphingolipid precursor upregulation or accumulation (i.e. ceramide).

Light microscopic analysis by *bis*-benzimide staining and DNA laddering by gel electrophoresis also revealed apoptotic cell death of DRG neurons exposed to the short chain ceramide analog C_2-ceramide or sphingomyelinase. The onset of ceramide induced apoptosis in DRG neurons was comparable to recent reports describing ceramide mediated programmed cell death in mesencephalic neurons and embryonic chick cerebral neurons (Brugg *et al*, 1996; Wiesner and Dawson, 1996). Nonspecific ceramide toxicity was excluded as the dihydroceramide analog of C_2-ceramide, which differs only by the absence of 4–5 *trans* double bond in the sphingosine moiety, had no effect on DNA integrity as determined by gel electrophoresis (Figure 6). Similar results were observed in TUNEL studies (data not shown). This analog has previously been shown incapable of inducing apoptosis and internucleosomal DNA digestion (Bielawska *et al*, 1993; Obeid *et al*, 1993). This result along with other findings presented here provide strong evidence that neurons exposed to suramin or agents that elevate intracellular ceramide levels (i.e. C_2-ceramide or sphingomyelinase) initiate a pathway leading to programmed cell death.

The observed time course of ceramide mediated cell death in DRG neurons is compatible with the activation of a regulated program involving new gene transcription and/or protein synthesis. The longer period for suramin mediated changes in nuclear integrity would correspond to the time needed to accumulate ceramide levels via defects in glycolipid metabolism sufficient to initiate a ceramide mediated cell death pathway. Alternatively, NGF withdrawal revealed the earliest signs of apoptotic cell death, an event described in earlier reports (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). Whether NGF withdrawal involves ceramide mediated cell death pathways has not been determined.

In this study, suramin induced accumulation of ceramide was measured by two independent assays. While a recent report challenges the reliability of ceramide measurement via the diacylglycerol kinase assay (Watts *et al*, 1997), similar ceramide levels were observed by both MS analysis and the diacylglycerol kinase assay in suramin treated neuronal cultures (Figure 7). Accumulation of ceramide was observed within 12 h of suramin treatment, consistent with recent reports describing ceramide changes sufficient to initiate a cell death pathway (Brugg *et al*, 1996; Wiesner

and Dawson, 1996). In both assays, significant accumulation of ceramide was observed before any morphological changes conforming to apoptotic cell death. These findings suggest that suramin mediated ceramide accumulation precedes and possibly initiates a cell death pathway rather than being a nonspecific consequence of drug induced cell damage.

The developing neurons used in this study are rapidly growing and elaborating axonal membranes. It is logical that glycosphingolipid synthesis will be maximally upregulated and that degradation pathways may be relatively inactive. Based on our studies using the pharmacologic inhibitor of ganglioside biosynthesis (Figure 4), we postulate that the rapid appearance of LIB is due to suramin induced production of ceramide with subsequent synthesis into complex glycosphingolipids (i.e. gangliosides). Furthermore, suramin induced LIB accumulation could be potentiated by a defect in trafficking of synthesized gangliosides to their correct target (unpublished observations). The expanding pool of gangliosides, accumulating from upregulation of precursor synthesis (i.e. ceramide) collect in lysosomes.

Finally, the role of ceramide in suramin mediated neuronal death presented in this study correlates with the drug's effect on cancer cells. A recent report described a similar mechanism of ceramide mediated cell death in a number of human cancer cell lines exposed to suramin (Gill and Windebank, 1997). It is proposed that suramin's cytotoxic and neurotoxic mechanisms of action are similar. Studies are underway to investigate the rate of glycolipid metabolism in the cancer cells in comparison to neurons.

Materials and Methods

Tissue culture

Embryonic day 15 rat pups were removed from the uterus under pentobarbital anesthesia. Pups were removed from the amniotic sac and held in Leibowitz L15 medium (GIBCO, Gaithersburg, MD), at 4°C during dissection. Each pup was decapitated and then DRG removed by microdissection. DRG were pooled and incubated with 0.25% trypsin in Hanks Balanced Salt Solution (GIBCO) for 30 min at 37°C. DRG were sedimented at 1000 r.p.m. for 5 min, supernatant removed, dissociated further with a small bore pipette, and resuspended in Eagle's Minimal Essential Medium (GIBCO) supplemented with 15% calf bovine serum (Hyclone, Logan UT) 10 ng/ml 2.5S NGF (Boehringer Mannheim, Indianapolis, IN), 0.6% glucose, 1.4 mM L-glutamine, and 50 μg/ml ascorbic acid. Dissociated DRG were plated onto ACLAR (American Chemical, Pleasant Gap, PA) dishes coated with ammoniated and air-dried rat tail collagen (Bornstein, 1958). Cells were grown overnight before the addition of antimetabolites (10 μM fluorodeoxyuridine; Sigma Chemical Co., St. Louis, MO) for 4 days. This yielded a predominantly neuronal culture.

Suramin (Mobay Chemical Corp., New York, NY) was constituted in standard medium and exposed to cultures for 12 h to 4 days. Cultures were also exposed to 5–10 μM of the short chain ceramide analog C_2-ceramide (Matreya Inc., Pleasant Gap, PA) as well as 5–10 μM of the structurally related lipid analog dihydroceramide (Matreya) which has not been documented to mediate apoptotic cell death (Obeid *et al*, 1993). Intracellular ceramide was also increased directly by incubating cultures with 300 mU/ml sphingomyelinase (*Staphylococcus aureus*; Sigma). Glucosylceramide synthase was inhibited by exposing cells to PDMP (*d,l-threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol), a potent and competitive inhibitor of the enzyme (Matreya) (Li and Ladisch, 1997). The inactive diastereomer *d,l-erythro*-PDMP was used for comparison studies.

Cholera toxin-β staining

Dissociated DRG cultures were exposed to 300 μM suramin for 12 h to 4 days. Cultures were fixed in 4% paraformaldehyde followed by incubation with cholera toxin-β subunit horseradish peroxidase conjugate, a specific marker for the monosialoganglioside GM_1 (Research Biochemicals International, Natick, MA). Positive staining was detected by the chromagen diaminobenzidine. Parallel suramin treated cultures were exposed to the lysosomal marker, Texas Red-dextran (Molecular Probes, Eugene, OR) overnight. Inclusion bodies/lysosomes were observed in live cells under light and fluorescence microscopy (568 nm excitation wavelength).

Electron microscopy

DRG cultures exposed to 300 μM suramin for various times were fixed *in situ* with Trump's fixative followed by 1 h postfixation in 1% OsO_4. Cultures were stained *en bloc* with 2% uranyl acetate, dehydrated and embedded in Spurr's resin. Sections (0.8 μm) were examined with a Philips CM10 transmission electron microscope.

Bis-benzimide staining

Nuclear fragmentation and condensation were examined with *bis*-benzimide (Hoechst 33258; Sigma) staining. Following treatment, cultures were washed twice with phosphate buffered saline and fixed for a minimum of 1 h in methanol:acetic acid (3:1). Cultures were again washed in phosphate buffered saline and mounted with a drop of 50% glycerol/50% 0.1 M Tris-HCl (pH 7.4) containing 1 μg/ml *bis*-benzimide. Cultures were stored in the dark for a minimum of 15 min and then observed under a fluorescent microscope using Hoechst optics.

DNA fragmentation

DNA from enriched DRG neuronal cultures was prepared by modification of a previous method (Batistatou and Greene, 1993). After treatment, cells were washed in phosphate buffered saline and pelleted. Cells were lysed in 200 μl of 50 mM Tris-HCl pH 9.0, 20 mM EDTA, 10 mM NaCl, 1.1% sodium dodecyl sulfate (w/v), and 10 mg/ml Proteinase K (GIBCO). Cells were incubated in lysis buffer for 48 h at 48°C. Samples were cooled at room temperature for 15 min and sedimented. Two hundered microliters of phenol chloroform was added to each pellet, vortexed, and sedimented again. Following a second phenol chloroform extraction, an aliquot containing 5 μg DNA was added to 50 μg/μl RNase A plus 3 μl gel loading buffer and incubated at room temperature for 1 h. Samples were electrophoresed in a 1.2% agarose gel and visualized with ethidium bromide.

Ceramide measurement

A modification of the method by Preiss and colleagues (Preiss *et al*, 1987) in which diacylglycerol kinase converts ceramide to ceramide-1-[^{32}P]phosphate was used. Briefly, lyophilized whole cell lysates of

enriched DRG neuronal cultures were solubilized in 20 μl of 7.5% n-octyl-β-D-glucopyranoside, 5 mM cardiolipin, and 1 mM diethelene-triaminepentaacetic acid solution by heating to 70°C, followed by sonication and vortex-mixing. The volume was then brought to 100 μl in a mixture containing 72 mM imidazole, 12.5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM EDTA, 0.4 mM diethylenetriaminepentaacetic acid, 50 mM LiCl, 50 μg/ml of diacylglycerol kinase and 1 mM [^{32}P]ATP (5 μCi). After 30 min at room temperature, phosphorylated lipids were extracted. Samples were spotted on high performance thin layer chromatography plates and developed in chloroform/methanol/acetic acid (65 : 15 : 5 by volume). Standards of ceramide-1-phosphate were used for identification of bands. The high performance thin layer chromatography plate was exposed to film and intensity of bands measured by optical densitometry.

Ceramide levels in suramin treated cultures were also measured by mass spectrometry (MS) as described previously (Gu *et al*, 1997; Watts *et al*, 1997). Neuron enriched DRG cultures were spun down and resuspended and sonicated in 1 ml of CH_3OH/H_2O/12M HCl (95 : 5 : 0.5; vol/vol), and 0.6 ml of ice-cold water was added. Lipids were prepared by partitioning them into 1 ml of $CHCl_3$, and the organic phases were recovered and dried in glass tubes. Two ml of $CHCl_3$ were added with polar lipids removed by small-scale silica gel chromatography, and a single fraction spanning the known elution positions for ceramides varying from C2 : 0 to C24 : 1 was collected and dried. Lipids were resuspended in 5 mM ammonium acetate in methanol with C2 : 0 ceramide (Matreya) added to 200 pM/μl as an internal standard. Samples (10 μl) were then infused into a PE-Sciex 365 triple quadropole mass spectrometer (Sciex, Toronto, Canada) by a Perkin Elmer 200 Autosampler, with a flow rate of 5 μl/min. Mass analysis was performed as described previously (Watts *et al*, 1997).

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