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Nerve growth factor and ceramides modulate cell death in the early developing inner ear

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SUMMARY

Regulation of normal development involves a dynamic balance of the mechanisms regulating cell division, differentiation and death. We have investigated the signalling mechanisms involved in regulation of the balance between cell proliferation and apoptotic cell death in the otic vesicle. The sphingomyelin pathway signals apoptosis for nerve growth factor upon binding to p75 receptors. It is initiated by sphingomyelin hydrolysis to generate the second messenger ceramide. In the present study, we show that nerve growth factor stimulates sphingomyelin hydrolysis and the concomitant ceramide release in organotypic cultures of otic vesicles. Both nerve growth factor and ceramide induce apoptotic responses to a different extent. Ceramide-induced apoptosis was suppressed by insulin-like growth factor-I which is a strong promoter of cell growth and morphogenesis for the developing inner ear. In contrast, ceramide-1-phosphate protected the explants from apoptosis induced by serum withdrawal but did not antagonise ceramide-induced cell death. This study suggests that sphingomyelin-derived second messengers might be key modulators of programmed cell death during development.

Key words: Apoptosis, Ceramide-1-phosphate, Insulin-like growth factor, Lipid messenger, Morphogenesis, Nerve growth factor, Neurogenesis, Otic vesicle, PCNA

INTRODUCTION

Programmed cell death is a physiological process during development (Raff et al., 1993; Jacobson et al., 1997). In the nervous system it is essential at early developmental stages for tissue morphogenesis and neurogenesis, before innervation (Glücksmann, 1951; Saunders, 1966). Apoptosis has been reported to be modulated by cytokines including insulin-like growth factor-I (IGF-I) and nerve growth factor (NGF). IGF-I is a potent suppressor of cell death in a variety of cell types but little is known about its role during early embryonic development (Harrington et al., 1994; O'Connor et al., 1997). NGF has been shown to both trigger apoptosis and promote cell survival depending on the cell type and stage of development (Xia et al., 1995; Nobes and Tolkovsky, 1995; Davies, 1997). Distinct actions of NGF can be achieved by selective binding to either trkA or p75 receptors (Frade et al., 1996).

The signalling pathways mastering apoptosis during early organogenesis are poorly characterised. Ceramide (Cer) generation by the action of sphingomyelinase has been proposed to be a potent inductor of programmed cell death. This pathway is turned on by a number of effectors including CD95 (Apo-1/Fas), tumor necrosis factor, ionizing radiation or chemotherapeutic agents (reviewed by Hannun, 1996), and has been extensively studied in the immune system (Cifone et al., 1994; Gill et al., 1994). Sphingomyelin/Cer turnover is also activated by NGF in primary cultures of postnatal oligodendrocytes (Casaccia-Bonnefil et al., 1996). Cer actions are exerted through the activation of specific kinase and phosphatase activities (Joseph et al., 1993; Dobrowsky et al., 1994). Other downstream targets for ceramides are phospholipase D, protein kinase C-ζ, Ras, Vav, Raf and the transcription factor NFκB (for reviews see Spiegel and Merril, 1996; Hannun, 1996). Cer can be converted to ceramide-1-phosphate (Cer-1-P) by the action of a calcium-dependent kinase (Dressler and Kolesnick, 1990). Cer-1-P has been reported to modulate cellular proliferation and the expression of proliferating cell nuclear antigen (PCNA) in fibroblasts (Gómez-Muñoz et al., 1995a, 1997).

The inner ear in vertebrates derives from the otic vesicle, which goes through a period of intense cell proliferation that is accompanied by morphogenesis. Cochleovestibular ganglion (CVG) cells form from the ventromedial aspect of the otic vesicle from where neuroblasts migrate and proliferate. Then, post-mitotic neuroblasts differentiate and start to extend processes towards their central and peripheral targets (Hemond and Morest, 1991). Both NGF and IGF-I have been reported to

modulate main biological processes in the developing ear. In the chick embryo, IGF-I is a potent mitogen for the epithelial cells of the otic vesicle, and it also induces cellular proliferation and neurite outgrowth in the CVG (León et al., 1995a; Y. León and I. Varela-Nieto, unpublished). IGF-I high affinity receptors are expressed in the inner ear, and inmunocytochemical studies indicate endogenous expression of this factor at embryonic day 2 and 3 (León et al., 1995a). NGF induces cell proliferation in the CVG but not in the otic vesicle (Represa and Bernd, 1989; Represa et al., 1991). NGF low affinity receptors, the p75 glycoprotein, are expressed in the otic vesicle and ganglion (von Bartheld et al., 1991; Wu and Oh, 1996).

The aim of this work is to study the role of sphingomyelin-derived metabolites in the regulation of cell survival and its relation with NGF and IGF-I. We report here that Cer and Cer-1-P antagonistically modulate cell survival in the otic vesicle. Addition of exogenous Cer or Cer-1-P to chick embryo organotypic cultures has opposite effects in the inner ear suggesting that the balance between these metabolites may contribute to determine cell fate and that this balance may be regulated by NGF and IGF-I.

MATERIALS AND METHODS

Materials

Recombinant NGF was from UBI (Lake Placid, NY). Chicken and human recombinant IGF-I were purchased from Gropep (Adelaide, Australia) and Boehringer Mannheim (Mannheim, Germany), respectively. Both factors are equipotent when assayed in organotypic cultures of chicken otic vesicles (Y. León and I. Varela-Nieto, unpublished observation).

Antibodies to PCNA were from Concepta Biosystems, SA (Barcelona, Spain). Cell permeable acetyl-Cer and octanoyl-Cer-1-P were from Matreya Inc. (Pleasant Gap, PA) and Calbiochem (San Diego, CA), respectively. Bacterial sphingomyelinase was from Sigma Chemical Co (St Louis, MO). [^{3}H]thymidine (49 Ci/mmol) and [^{3}H]palmitic acid (52 Ci/mmol) were from Amersham Ltd (Little Chalfont, UK).

Preparation of organotypic cultures.

Fertilised eggs (Granja Rodríguez Serrano, Salamanca, Spain) were incubated at 38°C in a humidified atmosphere and were staged according to the criteria of Hamburger and Hamilton (1951). Embryos were removed from eggs and placed in 35 mm Petri dishes (NUNC, Roskilde, Denmark) containing PBS. Otic vesicles were dissected from chick embryos corresponding to stage 18 (embryonic day 2.5) using a dissection stereomicroscope and transferred into four-well culture plates (NUNC). The standard culture medium consisted of serum-free M199 medium with Earle's salts (Biowhitaker, Walkersville, MD) supplemented with 2 mM glutamine (Biowhitaker), and antibiotics (penicillin, 50 i.u./ml and streptomycin, 50 μg/ml) (Biochrom, Berlin, Germany). Fetal bovine serum was purchased from Biowhitaker. Incubations were carried out at 37°C in a water-saturated atmosphere containing 5% CO_2. Otic vesicles were made quiescent by incubation in serum-free medium for 24 hours before the different experiments were performed.

Immunoblotting

For western blotting, otic vesicle explants were homogenised in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer with 1 mM phenylmethylsulfonyl fluoride and frozen immediately. Gels were loaded with solutions containing equal amounts of protein, typically one otic vesicle per condition. Otic vesicle proteins were resolved by using 10% SDS-PAGE and then transferred onto PVDF membranes (Dupont-NEN, Boston, MA). Filters were blocked with Tris-buffered saline containing 5% (w/v) non-fat dried milk, and incubated with the primary specific antibody. Filters were subsequently washed and incubated with the corresponding secondary antibody conjugated with peroxidase. Bound peroxidase activity was visualised by chemiluminescence (Dupont-NEN) and quantified by densitometry.

Proliferative cell nuclear antigen (PCNA) is a non-histone nuclear protein of 36 kDa that is associated with regulation of the cell cycle, and it is essential for DNA synthesis (Bravo and MacDonald-Bravo, 1987). Mouse monoclonal anti-PCNA was used at a dilution of 1:2,000. Secondary antibodies conjugated with peroxidase were purchased from Bio-Rad, CA.

Otic vesicle labelling and determination of [^{3}H]sphingomyelin hydrolysis

Otic vesicles were isolated from stage 18 chick embryos and labelled with 25 μCi/ml of [^{3}H]palmitic acid for 24 hours, in the presence of 1% fetal bovine serum. Otic vesicles were washed with PBS, placed on culture dishes in serum-free medium at 37°C for a further period of 3 hours and stimulated with either sphingomyelinase or NGF as indicated. Cellular lipids were extracted and labelled Cer was isolated as indicated (Gómez-Muñoz et al., 1997). Briefly, lipids were extracted according to the method of Bligh and Dyer (1959) except that phases were separated by adding 2 M KCl in 0.2 M H_3PO_4 instead of water. The organic phase was dried under N_2 and the lipid extracts were applied onto thin-layer plates (silica Gel G60 t.l.c., Merck, Darmstadt, Germany) which were developed twice with chloroform/methanol/acetic acid (9:1:1, by vol.). The material which commigrated with cold Cer standards was scraped and extracted with 1 ml methanol at 37°C. Radioactivity associated with the spots was determined by scintillation counting. Alternatively, labelled sphingolipids were resolved on t.l.c. plates as described above but dried plates were impregnated with En^{3}Hance (Amersham) and subjected to autoradiography.

Determination of [^{3}H]thymidine incorporation in otic vesicle cultures

Otic vesicle explants were cultured in medium containing [^{3}H]thymidine (0.2 μM, 10 μCi/ml) for 24 hours. Incubations were carried out at 37°C in a water-saturated atmosphere containing 5% CO_2. Otic vesicles were then individually rinsed three times with PBS. Explants were then treated with 10% trichloroacetic acid and the radioactivity incorporated determined by liquid scintillation counting (OptiPhase 'HiSafe' liquid scintillation cocktail. Wallac, Turku, Finland).

Cell death determination by immunoassay and TUNEL staining

Quantification of cell death was performed by using the Cell Death Detection ELISA from Boehringer Mannheim, which is based on the detection of histone-associated DNA fragments (mono-and oligo-nucleosomes) in the cytoplasm of cells. Cultured otic vesicles were homogenized and the cell extracts subjected to enzyme-linked immunosorbent assay (ELISA) determination basically as indicated by the manufacturer.

Distribution of apoptotic cells in the otic vesicle was determined by TUNEL staining with the modifications reported (Blaschke et al., 1996) and adapted to whole organ labelling (B. Díaz, F. de Pablo and E. J. de la Rosa, unpublished data). Fragmented DNA was labelled with Biotin-dUTP by the terminal transferase reaction according to the manufacturer's instructions (Boehringer Mannheim). Apoptotic nuclei were visualised with Cy2-Streptavidin (Amersham) and analysed with a MRC1024 Bio-Rad confocal microscope.

All data are expressed as the means ± s.e.m. unless stated otherwise. Statistical evaluation was by conformity *t*-test.

RESULTS

Opposite effects of exogenous Cer and Cer-1-P in otic vesicle cultures

In the following experiments Cer and Cer-1-P were added to quiescent otic vesicles and tested for cell proliferation. Treatment of quiescent organotypic otic vesicle cultures with 5 μM Cer for 24 hours induced disruption of the otic vesicle (Fig. 1A) and no changes in PCNA expression (Fig. 1B). By contrast, addition of 25 μM Cer-1-P caused a modest increase in explant size of about 1.7-fold, which was concomitant with the induction of PCNA levels (Fig. 1B). Samples treated with 1 nM IGF-I are shown for comparison. Cer blocked the effects of Cer-1-P on PCNA expression even at a concentration of Cer-1-P 5 times higher than those of Cer. In contrast, 1 nM IGF-I counteracted to large extent the effects of Cer (Fig. 1B).

Experiments on [^{3}H]thymidine incorporation into DNA indicated that explants treated with Cer, did not incorporate labelling when compared with control quiescent otic vesicles (Fig. 2A, filled symbols), whereas those treated with Cer-1-P incorporated [^{3}H]thymidine up to 1.8-fold over explants kept in free-serum medium (Fig. 2A, open symbols). Both effects were dose-dependent. Mitogenic lipids such as lysophosphatidic acid (10-50 μM), sphingosine (5-25 μM), sphingosine-1-phosphate (5-10 μM) or sphingosyl-phosphorylcholine (5 μM), had no significant effect on [^{3}H]thymidine uptake by the otic vesicles in two different experiments performed in quadruplicate (data not shown). Higher doses tested were cytotoxic in this system, as reported for other cell types (Spiegel and Merrill, 1996). Furthermore, addition of up to 10 units/ml of phospholipase D to the cultures did not induce cell growth (data not shown). Therefore, it appears that Cer-1-P is specific for the stimulation of [^{3}H]thymidine uptake in this system. A similar approach was used to quantify IGF-I cytoprotective activity. IGF-I was able to suppress Cer effects which in turn partially counteracted IGF-I-stimulation of cell growth (Fig. 2B).

These data suggest that both lipid messenger molecules have opposite effects in otic vesicle development. Whereas Cer data could be explained by an increase in apoptotic cell death in the cultures, the role of Cer-1-P was less clear. Was it protecting otic vesicle explants from starvation-induced cell death, or was it a low-potency proliferative agonist? To quantify the effects on cell death resulting from the addition of Cer or Cer-1-P, soluble nucleosomes were measured in otic vesicle extracts by using a Cell Death Detection ELISA kit. Mono-and oligonucleosomes are present in the cytoplasm of cells undergoing apoptotic cell death, but not in necrotic cells (Collins and López Rivas, 1993). As shown in Table 1, Cer was a potent inductor of apoptosis in otic vesicle organotypic cultures and Cer-1-P prevented cell death. Again, IGF-I was able to suppress Cer-induced apoptosis, however, Cer-1-P did not. These results are in contrast with those obtained in other

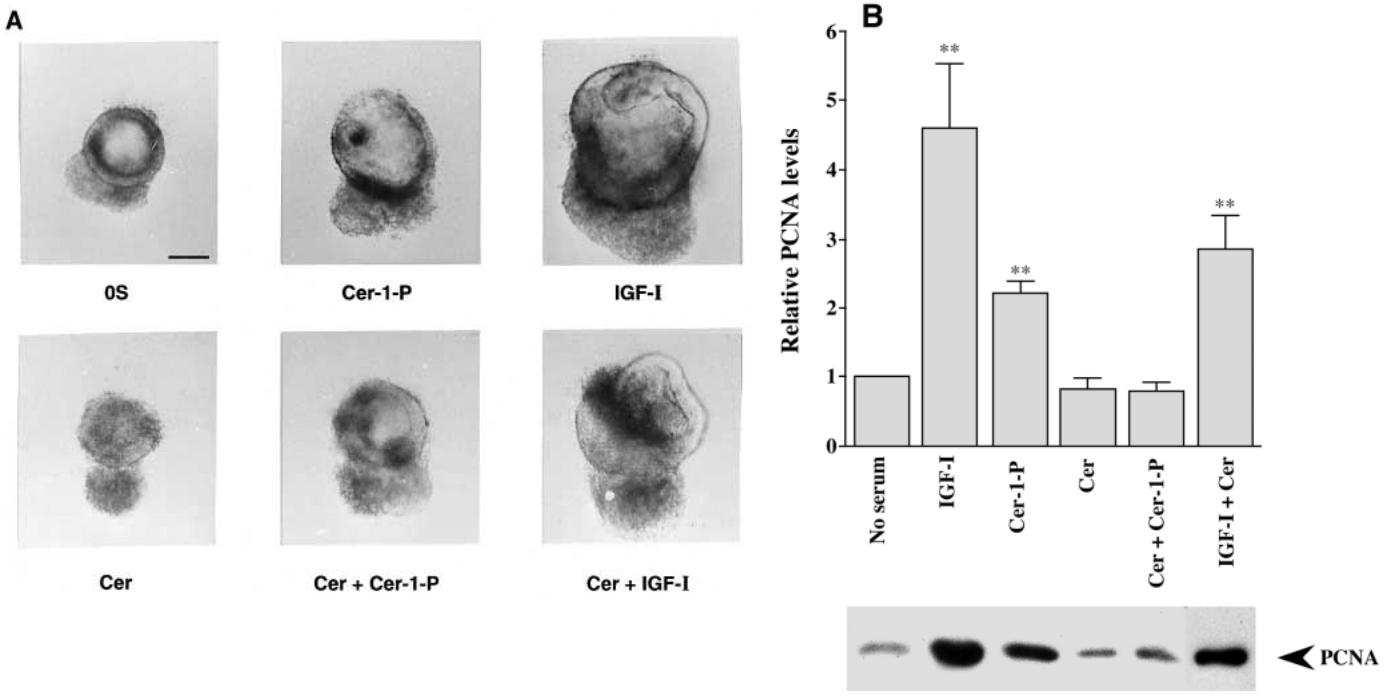

Fig. 1. (A) Effects of Cer and Cer-1-P on explanted otic vesicles. Otic vesicles were explanted from stage 18 chick embryos and cultured for 24 hours in the absence of serum (0S), or with either 5 μM Cer, 25 μM Cer-1-P, 1 nM IGF-I or combinations of these agonists as indicated. The photographs of the explants shown are representative of at least five different experiments performed in quadruplicate. Bar, 100 μm.
(B) Expression of proliferating cell nuclear antigen (PCNA). Otic vesicles were incubated for 24 hours in serum free medium or in the presence of the different additives as indicated. Lysates were analysed by SDS-PAGE and western blotting as described in Materials and Methods. PCNA spots were quantified by densitometry. Average densitrometrical values are plotted on bars. Symbols are as above. Results are expressed relative to the control value (no serum, 0S) and they are given as means ± s.e.m. of five independent experiments performed in triplicate. The significance of the differences between incubations under the different conditions is as follows: *P<0.05 and **P<0.001. A representative blot of otic vesicles incubated under the different conditions is shown in the lower layer.

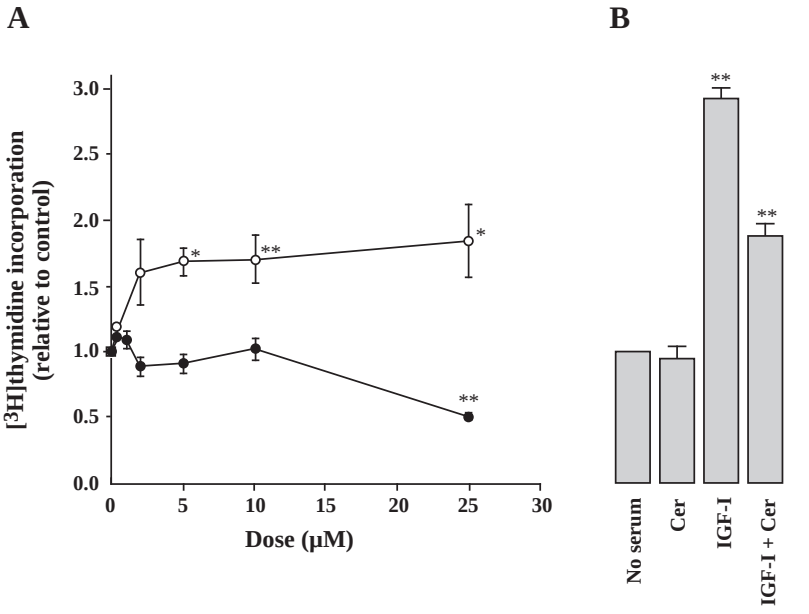

Fig. 2. (A) Effects of Cer-1-P and Cer on [^{3}H]thymidine uptake by otic vesicle cultures. Otic vesicles were cultured in serum-free medium containing [^{3}H]thymidine in the presence of Cer-1-P (open symbols) or Cer (filled symbols) at the concentrations that are indicated. Results are expressed relative to the control value. The values are given as mean ± s.e.m. of four experiments performed in quintuplicate. (B) Effects of the addition of IGF-I on [^{3}H]thymidine uptake. Quiescent otic vesicles were cultured for 24 hours with no serum, 1 nM IGF-I or with 5 μM Cer in the presence or absence of 1 nM IGF-I. Data are expressed as mean ± s.e.m. of at least four different experiments with an average of five explants per data point. Statitiscal significance is as follows: *P<0.05 and **P<0.001 vs no serum.

systems. Cer has been reported to block IGF-I action by reducing tyrosine phosphorylation of the insulin receptor substrate-1 (Kanety et al., 1996). In the otic vesicle, however, IGF-I is a survival factor able to block Cer-induced apoptosis suggesting that in this system there are alternative routes of signalling.

Characterisation of an endogenous sphingomyelin pathway

At this point it was important to assess the physiological relevance of the observed effects of Cer and Cer-1-P. To this end we performed the following experiments. Otic vesicle explants were labelled with [^{3}H]palmitic for 24 hours. At the end of the labelling period, different doses of sphingomyelinase were added and the incubation continued for a further period of 2 hours. Reaction was stopped by freezing the samples in dry ice and lipids extracted and separated by t.l.c. (Gómez-Muñoz et al., 1997). Finally, the amount of [^{3}H]Cer released was quantified. Fig. 3A shows a typical chromatographic profile of labelled lipids which are compared with lipid standards. The profile indicates the presence of sphingomyelin (SM in Fig. 3A) and Cer in the inner ear. Fig. 3B shows that otic vesicle endogenous sphingomyelin is sensitive to sphingomyelinase hydrolysis. Sphingomyelinase is implicated in the generation of Cer that leads to DNA fragmentation (Wiegmann et al., 1994). Cer release is dose-

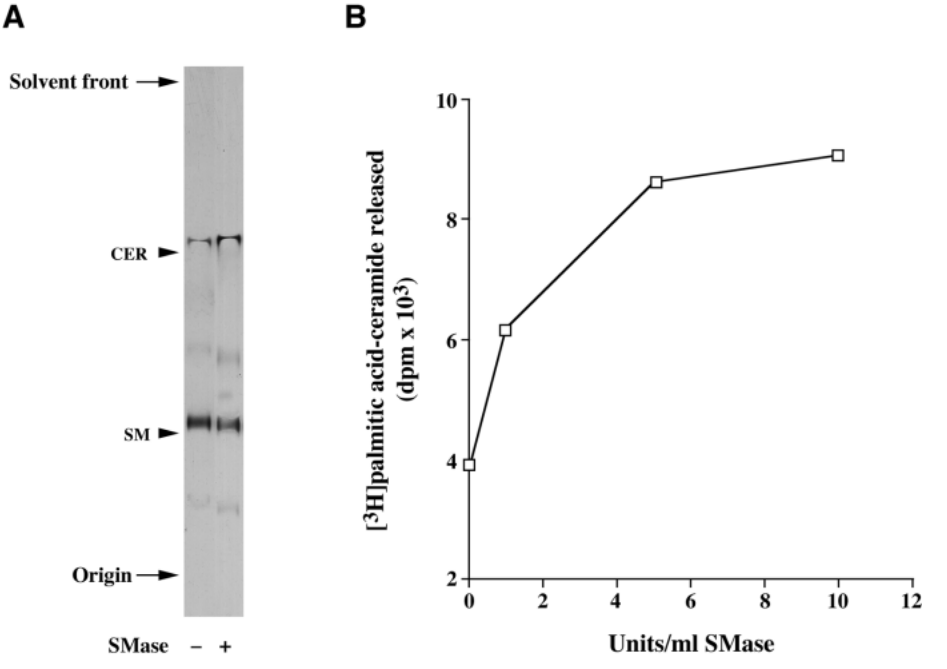

Fig. 3. (A) Labelling of sphingomyelin and Cer with palmitic acid. Five otic vesicle explants were labelled for 24 hours with [^{3}H]palmitic acid and treated or not with sphingomyelinase (SMase) for three hours, after which total cellular lipids were extracted and both sphingomyelin and Cer were purified by sequential t.l.c. procedures as described in Materials and Methods. The data are taken from a representative experiment performed in triplicate. Total number of different experiments performed was four. The position of marker lipids are indicated: CER, ceramide; SM, sphingomyelin. (B) Dose-dependency of sphingomyelinase-stimulated sphingomyelin hydrolysis in isolated otic vesicles. 5 explants were labelled as above with [^{3}H]palmitic acid before incubation with increasing doses of sphingomyelinase (SMase) for three hours. Radiolabelled Cer was isolated by t.l.c. and the amount of radioactivity associated with the cold Cer carrier was assessed as described in Materials and Methods. The results are representative of two experiments performed in triplicate.

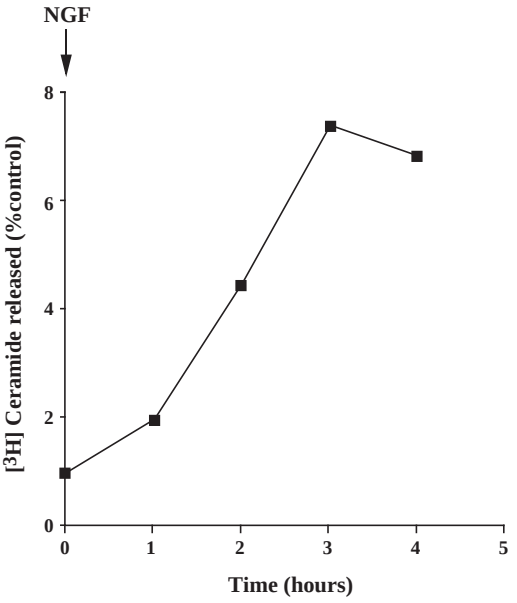

Fig. 4. NGF stimulates generation of Cer. Otic vesicles were labelled with 25 μCi/ml of [^{3}H]palmitic acid for 24 hours. Then, the vesicles were incubated in serum-free medium containing 4 nM NGF for the periods (hours) that are indicated. Lipids were analysed as described in Materials and Methods. Results are from a representative experiment and they were confirmed in two independent experiments that were performed in quintuplicate. The release of [^{3}H]Cer after 3 hours of incubation with NGF was about 7-fold higher than control values.

dependent and it reached saturation at a concentration of sphingomyelinase of about 5 units/ml.

NGF-induced generation of endogenous Cer

The next step was to test whether sphingomyelin hydrolysis could be stimulated by physiological factors. NGF was the preferred candidate since it has been shown to induce Cer production (Dobrowsky et al., 1994; Casaccia-Bonnefil et al., 1996) and cell death in the developing retina (Frade et al., 1996). In addition, patches of expression of p75 receptor have been reported in the vesicular epithelium (Wu and Oh, 1996). These results are consistent with the idea that a restricted pool of cells may be able to sense NGF and enter apoptosis. NGF (4 nM) induced the generation of endogenous Cer (Fig. 4). Cer release was maximal after three hours of incubation. The ability of NGF to induce apoptosis in otic vesicle cultures was measured in parallel and rendered a value of 165±11 (n=14, P<0.001) that should be compared with data presented in Table 1.

To further study whether NGF modulates cell death in the inner ear we used the TUNEL assay. This approach is indicated to analyse DNA fragmentation in individual apoptotic cells in a population. As shown in Fig. 5, NGF induced apoptotic cell death in three particular areas (D-G): (i) at the ventromedial wall of the otocyst, where the CVG is being formed (E,F); (ii) within the CVG (D); and (iii) at the dorsal wall of the otocyst, which corresponds with the area of development of the endolymphatic duct (G). Exogenous Cer induced cell death but with a less restricted pattern (H-K). Compare H with control explant (A) cultured in no serum, and H with D. However, cell death was more intense in the areas mentioned above, suggesting that those cell populations are somehow primed to die. Finally, Cer-1-P suppressed starvation-induced cell death (B and C) as compared with control quiescent otic vesicles cultured in the absence of serum (A).

DISCUSSION

Our work shows that: (i) exogenously added Cer is a potent activator of apoptosis in the otic vesicle; (ii) Cer-1-P is a cytoprotector; (iii) Cer-induced cell death is suppressed by IGF-I that is a survival factor for the developing inner ear; (iv) NGF stimulates the sphingomyelin/Cer turnover in the otic vesicle; and (v) NGF induces apoptosis in restricted areas of the otic vesicle.

Apoptosis is an important physiological mechanism for regulation of development (Jacobson et al., 1997). Signals regulating cell death will cooperate with those managing cell division and differentiation to control normal development and tissue function in complex networks. Cells at certain stages undergo apoptosis in response to a number of pharmacological or physiological stimuli such as NGF (Dobrowsky et al., 1994; Frade et al. 1996), whereas other growth factors prevent the onset of apoptosis. Insulin attenuates apoptosis in the chick embryo during neurulation (Morales et al., 1997) and IGF-I has been shown to act as a survival factor in various cell types including neural cells (Rodríguez-Tarduchy et al., 1992; Matthews and Feldman, 1996; Párrizas and LeRoith, 1997). During the early stages of inner ear development IGF-I has been proposed to be an endogenous physiological factor promoting cell proliferation (León et al., 1995a) and NGF has been reported to be a proliferative signal for the attached CVG (Represa and Bernd, 1989; Represa et al., 1991). We have studied the signalling mechanisms mediated by lipids involved in the regulation of apoptotic cell death and survival in organotypic cultures of the developing ear.

Sphingomyelinases hydrolyze sphingomyelin to form phosphocholine and Cer. Since Cer functions as a second messenger in the induction of apoptosis, these enzymes have been implicated in the regulation of cell death (Spiegel et al., 1996; Hannun, 1996). We report here that Cer is a potent inductor of apoptosis in the developing inner ear which is consistent with previous work (Obeid et al., 1993; for a review see Testi, 1996). On the contrary, sphingosine, sphingosine-1-phosphate and Cer-1-P, which are metabolites of Cer, have been reported to induce cell proliferation (Gómez-Muñoz et al.,

Table 1. Cell death in E 2.5 otic vesicles

No serum	(n=12)	100
Cer	(n=13)	145±5*
Cer-1-P	(n=12)	88±5†
Cer+Cer-1-P	(n= 9)	148±9*
IGF-I	(n=13)	80±3†
IGF-I + Cer	(n=10)	93±5‡

Otic vesicles were isolated and treated with ceramide (5 μM, Cer), ceramide-1-phosphate (25 μM, Cer-1-P) or IGF-I (1 nM). Soluble nucleosomes were quantified in single otic vesicle explants. For each experiment the absorbance obtained in otic vesicles cultured in the absence of serum was given a value of 100; n, the total number of explants tested in at least 5 different experiments. Statistical significance is as follows: *P<0.001, †P<0.05, vs no serum and ‡P<0.001 vs Cer.

1995a, 1997; Spiegel et al., 1996). Thus, it has been proposed that cell fate might be determined by a dynamic equilibrium between the production of Cer and the generation of mitogenic lipids (Gómez-Muñoz et al., 1995b, 1997; Cuvillier et al., 1996). Here we show that during embryonic development Cer and Cer-1-P have opposite effects on cell survival in the otocyst. A noteworthy difference with the cell culture systems tested before is that sphingosine and other lipid metabolites

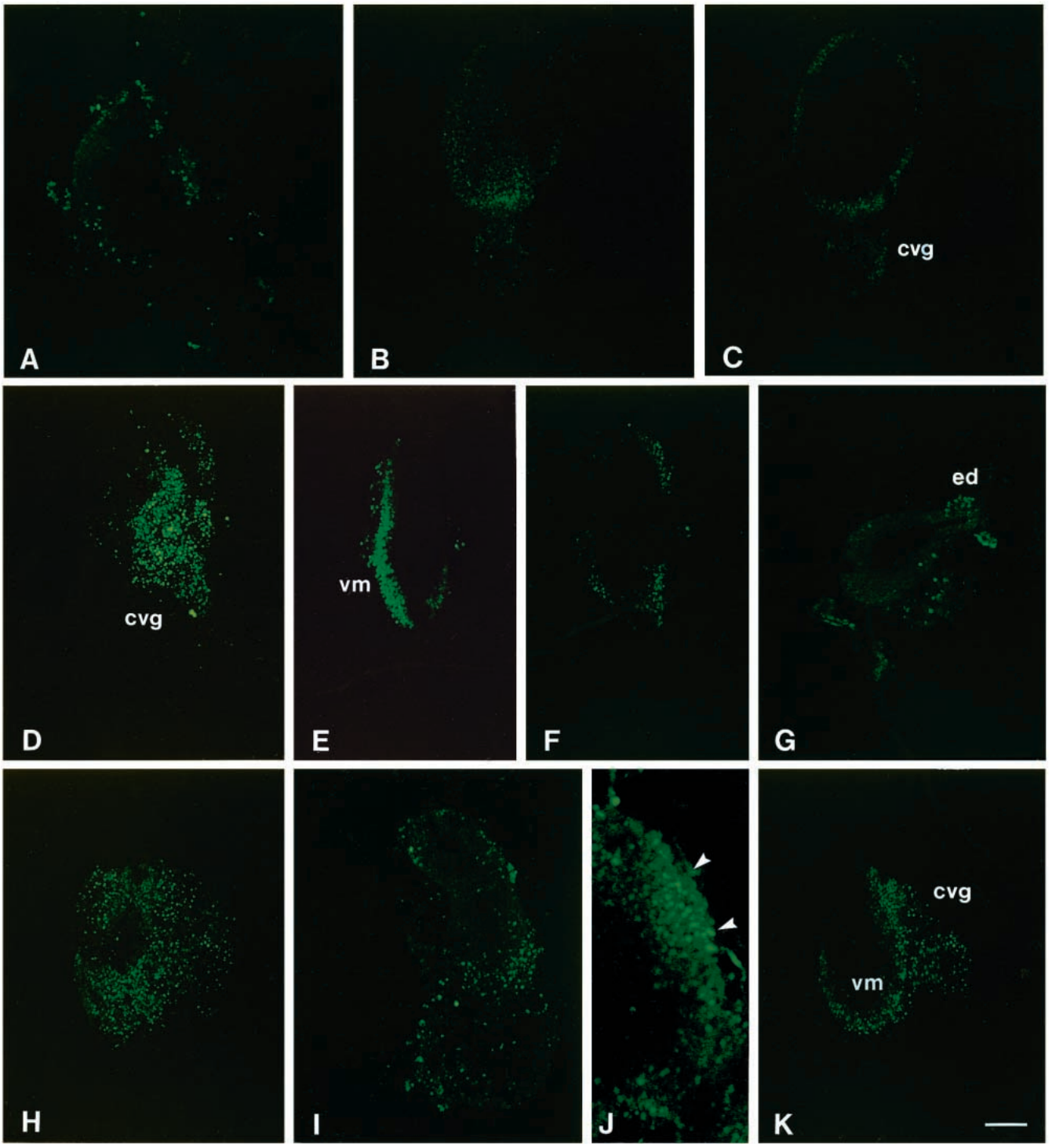

Fig. 5. Distribution of apoptotic cells in the otocyst. Apoptotic cell death was revealed by in situ DNA-end labelling technique (TUNEL protocol) in cultures of otocysts from embryonic day 2.5. Otic vesicles were isolated and grown for 8 hours in serum-free medium (A), 25 μM Cer-1-P (B, C), 4 nM NGF (D, E, F, G) or 5 μM Cer (H, I, J, K). (J) Cer-treated otic vesicle at a higher magnification showing a hot spot of cell death in the ventromedial wall. Arrowheads in J, typical rounded-shape and brighter color TUNEL-labelled cells.(B,D,H) Single projections of the whole vesicle whereas all the other panels show 2.5 μm optic sections. Bar, 50 μm (A-I, K); 10 μm (J). cvg, cochleovestibular ganglia; vm, ventromedial epithelium; ed, endolymphatic duct.

including lysophosphatidic acid, sphingosine-1-phosphate and sphingosylphosphorylcholine had no detectable effects. Cer-1-P has been reported not to be significantly metabolized to sphingosine or sphingosine-1-phosphate in fibroblasts (Gómez-Muñoz et al., 1995b). Natural Cer-1-P does not affect the expression of c-*fos* or c-*myc* nor does it exhibit a clear crosstalk with other signalling pathways relevant for cell proliferation (Gómez-Muñoz et al., 1997). However, Cer-1-P modulates the levels of PCNA that it is associated with progression of cell cycle and with DNA repair (Bravo and MacDonald-Bravo, 1987; Dietrich, 1993). Our data indicate that Cer-1-P is not a mitogen but a cytoprotector for otic vesicle explants acting as a suppressor of cell death upon withdrawal of serum. Hence, at least during development, the role of endogenous Cer-1-P might be the maintenance of cell survival probably by a mechanism involving upregulation of PCNA.

The p75 neurotrophin receptor is structurally related to tumor necrosis factor receptor and Fas family (Beutler and van Huffel, 1994). These receptors couple to at least two parallel signalling pathways to drive either apoptotic cell death or activation of the transcription factor NFκB (Carter et al., 1996). The presence of a death domain motif has been shown in p75 NGF receptor (Rabizadeh and Bredesen, 1994) which upon neurotrophin binding activates the sphingomyelin pathway to produce Cer (Dobrowsky et al., 1994; Cassacia-Bonnefil et al., 1996). The presence of p75 NGF receptor has been extensively reported at different stages of inner ear development in several animal species including chicken (von Bartheld et al., 1991; Schecterson and Bothwell, 1994). Wu and Oh (1996) recently proposed that the restricted pattern of expression of p75 receptors within the otocyst epithelium is associated with presumtive sensory organ areas. Furthermore, recent work by Fekete and colleagues (Fekete et al., 1997) has provided evidence that cell death is a major player in morphogenesis of the semicircular canals. Our data indicate that NGF induces cell death in both the otic vesicle and the attached sensory ganglia, the CVG. NGF-induced apoptosis is apparent in three main areas which correlate well with earlier morphological descriptions of cell death in chicken (Valdecasas et al., 1977; Ard and Morest, 1984). A dorsal area where the endolymphatic duct develops, a ventromedial area where migration of placodial cells occurs pari pasu with early histogenesis of the CVG (Li et al., 1987), and finally the CVG itself which was the area with most abundant apoptotic nuclei. Apoptosis observed at the ventromedial area may be implicated in the control of the number of neuroblast precursors as suggested by earlier work (Ard and Morest, 1984). These results suggest a role for NGF and NGF-induced apoptosis in the early morphogenesis and neurogenesis of inner ear. Hence, in addition to its contribution in maintaining normal cell numbers, programmed cell death may also contribute to the formation of the endolymphatic duct and to the differentiation of the CVG. We also show that IGF-I in addition to promote cell division acts by protecting the cells of the otocyst from apoptosis. At this stage of development there are specific areas within the otic vesicle epithelium, the ventral and medial aspects, with higher mitotic activity (Represa et al., 1988; Alvarez et al., 1989; De la Pompa et al., 1995). The pattern of mitotic activity coincides with that of c-*fos* expression (León et al., 1995b). Therefore, the otic vesicle consists of an epithelium morphologically homogeneous, but it has regional specificity and possibly cell fate specificity (reviewed by Torres and Giraldez, 1997).

We propose that NGF and IGF-I may regulate normal growth of the otic vesicle via induction of regionally restricted areas of cell death and cell proliferation.

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