Ceramide-induced cell death/survival in murine osteoblasts

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Abstract

Programmed cell death (PCD) or apoptosis is a naturally occurring cell suicide pathway induced in a variety of cell types. We determined whether ceramide treatment contributes to reduced cell viability and increased PCD in primary osteoblasts and the signalling pathways that are involved. Cell viability was determined by the 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide assay. We found that C2-ceramide (≤10⁻⁷ M) promoted osteoblast viability, whilst concentrations ≥2×10⁻⁶ M significantly reduced osteoblast viability in a dose- and time-dependent manner. The effect of ceramide on cell viability was specific since C₂-dihydroceramide had no effect. Increasing intracellular ceramide levels with either sphingomyelinase (SMase) or an inhibitor of ceramide metabolism also increased osteoblast apoptosis. Ceramide-induced PCD in osteoblasts was determined by nuclear appearance and DNA fragmentation. PCD was induced by both C₂-ceramide and SMase. The ability of ceramide (5×10⁻⁸ M) to promote osteoblast survival was prevented by a general protein kinase C (PKC) inhibitor and by a PKC δ inhibitor, whilst osteoblast survival was enhanced in the presence of a protein phosphatase 1 (PP1) inhibitor. Phosphatidylinositol-3 kinase (PI3K) inhibitors had no effect on osteoblast survival. The ability of ceramide (5×10⁻⁵ M) to induce apoptosis was prevented by the inhibitors of PP1 and PKC δ, whilst the general PKC and PI3K inhibitors had no effect on it. Our findings suggest that ceramide signals osteoblast survival and apoptosis through different intracellular pathways, and that alteration in the intracellular levels of ceramide may play an important role in bone remodelling.


Introduction

The skeleton is a highly specialized and dynamic organ that undergoes continuous regeneration, a process termed remodelling. This process is responsible for the complete regeneration of the skeleton every 10 years. Remodelling involves the removal of bone by osteoclasts and the formation of new bone by osteoblasts. Up to 65% of osteoblasts that originate at the remodelling site die by apoptosis, or programmed cell death (PCD), a process common to several regenerating tissues (Jilka et al. 1998). The majority of surviving osteoblasts become osteocytes, buried within the bone matrix, which probably play a role in the coordination of further remodelling events, including repair of skeletal microdamage (Weinstein & Manolagas 2000). An increase in osteoblast and/or osteocyte apoptosis would therefore be expected to compromise skeletal integrity, and may contribute to the pathogenesis of bone loss and fragility fractures associated with sex hormone deficiency and glucocorticoid excess (Weinstein et al. 1998). The prevention of osteoblast cell death may be a useful treatment strategy for diseases such as osteoporosis, osteoarthritis and periodontitis.

Survival of osteoblasts can be promoted by growth factors (GFs) that are normally released by neighbouring cells or from the bone matrix. For example, basic fibroblast GF (FGF) and insulin–like growth factors (IGFs) 1 and 2 prevent osteoblast apoptosis (Hill et al. 1997). In contrast, tumour necrosis factor α (TNFα) which is a mediator of inflammatory bone loss induces osteoblast apoptosis (Hill et al. 1997), possibly through the generation of ceramide (Kitajima et al. 1996).

Ceramide, a lipid second messenger that increases the cellular oxidative state, has been implicated as a key molecule in the apoptotic process initiated by several different stimuli including trophic factor withdrawal and exposure to proinflammatory molecules (Kolesnick & Kronke 1998). Cellular ceramide synthesis increases in response to stress or death signals (Tepper et al. 1995). One pathway of ceramide formation involves sphingomyelin hydrolysis by either neutral sphingomyelinase (nSMase) or acidic sphingomyelinase (aSMase; Testi 1996); both enzymes are involved in several cell death programmes (Kolesnick & Kronke 1998). Intracellular levels of ceramide can also be modulated by de novo synthesis, or by the regulation of ceramide degradation (Hannun 1996).

Information regarding the effects of ceramide in cells of skeletal origin is limited and conflicting, which may be either killing or protecting cells depending on the experimental paradigm. The proapoptotic agent TNFα has been reported...
to induce osteoblast cell death, and ceramide was implicated in the process (Kitajima et al. 1996), but conversely ceramide has been shown to be mitogenic in MC3T3-E1 osteoblast cells (Takeshita et al. 2000).

Growing importance attributed to sphingolipid signalling in the control of cell survival, differentiation and death led us to examine the effects of ceramide on osteoblast survival and death. We also studied the relationship between sphingomyelin cycle activation and the signalling pathways involved in these responses.

Materials and Methods

Reagents

Terminal deoxynucleotidyl transferase, biotinylated dUTP and streptavidin fluorescein were purchased from Boehringer Mannheim GmbH. 3-(4,5-dimethyl-thiozol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), SMase, DL-threo-dihydrosphingosine, C2-ceramide, β-glycerophosphate, ascorbic acid (AA) and all cell culture reagents were purchased from Sigma Chemical Co. D-ascorbic acid (AA) and all cell culture reagents were purchased from BIOMOL (Plymouth, PA, USA). C2-ceramide and DL-threo-dihydrosphingosine were never exceeded 0.05%.

Preparation of osteoblasts from neonatal mouse calvariae and culture

Calvarial osteoblasts were prepared and characterized as described previously (Heath et al. 1984). Briefly, neonatal mouse calvariae were dissected free from adherent soft tissue, washed with Ca$_2^+$- and Mg$_2^+$-free Tyrode’s solution (10 min) and sequentially digested with 1 mg/ml trypsin (10 min), 2 mg/ml dispase (30 min) and 2 mg/ml collagenase (twice, 30 min each time). Cells released by the last two collagenase digestions were washed, and grown in an α-modified MEM (α-MEM) containing 10% FBS and antibiotics for 4 days before use. All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO$_2$–95% air. Cultures were monitored every day to establish the stage of confluence under an inverted phase microscope (Nikon, Tokyo, Japan).

For the differentiation of osteoblasts into mature cells, cultures were grown in 5 µg/ml AA containing medium for 6–8 days before use, and the effects of C2-ceramide were assessed as described below. To examine the effects of C2-ceramide on mineralizing osteoblasts, cells that had been grown in AA for 8 days were cultured in the presence of 10 mM β-glycerol phosphate and AA for an additional 6 days.

Cell survival assays

MTT assay Cell survival was assessed by the MTT assay as described previously (Mosmann 1983). The murine osteoblasts were seeded in 96-well plates (5×10$^3$ cells/well) in 100 µl of α-MEM with 5% FCS for 2 h to permit the adhesion of osteoblasts. Thereafter, medium was replaced with α-MEM containing 1% FCS and thymidine (10$^{-3}$ M) to block cell proliferation. To determine the effects of C2-ceramide on differentiated and matrix-mineralizing osteoblasts, AA (50 µg/ml) was added to induce differentiation until day 12. For the mineralizing experiments, β-glycerophosphate (4-4 nmol/l) was also added from day 12 to 18. The medium was then replaced with α-MEM containing 1% FCS and thymidine (10$^{-3}$ M) to block cell proliferation. MTT was dissolved in PBS at a concentration of 5 mg/ml and sterilized by passage through a 0.22-µm filter. This stock solution was added (one part to ten parts medium) to each well of a 96-well tissue culture plate, and the plate was incubated at 37 °C for 4 h. Viable cells with active mitochondria cleave the tetrazolium ring into a dark blue formazan reaction product. Acid-isopropanol (400 µl of 10 M HCl in 100 ml isopropanol) was added to all the wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature, to ensure that all the crystals were dissolved, the plates were read on a microplate reader at a wavelength of 570–630 nm. A standard curve was set up using 200–50 000 cells/well, and the absorbance was found to be directly proportional to the number of cells over this range. The percent survival was defined as

$\frac{\text{experimental absorbance} - \text{blank absorbance}}{\text{control absorbance} - \text{blank absorbance}} \times 100$,

where the control absorbance was the value obtained for 5000 cells/well, which is the number of cells plated at the start of the experiment, and the blank absorbance was the value obtained for the wells containing the medium and MTT without the cells.

Identification of apoptotic osteoblasts

Quantitative DNA fragmentation ELISA Cells were grown in 12-well culture plates for DNA fragmentation. After treatment, floating cells were discarded, and the attached cells were washed twice with Dulbecco’s PBS. Cells were lysed and centrifuged to remove the nuclei. An aliquot of the nuclei-free supernatant was placed in streptavidin–coated wells. A mixture of anti-histone biotin and anti-DNA peroxidase–conjugated antibody for DNA fragmentation was added to the wells and incubated for 2 h at room temperature. After incubation, the sample was removed, and the wells were washed three times with incubation buffer. After the final wash was done, 100 µl of the peroxidase substrate, 2,2'-azino-di(3-ethylbenzthiazoline-sulphonate), were added to the wells and incubated at room temperature. The absorbance was read at 405 nm using a microplate reader. Results were expressed as absorbance 405 nm/min per mg protein.
Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling DNA cleavage was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) reaction as described (Gavrieli et al. 1992). Primary osteoblasts were cultured in labtech chamber slides (Nunc) in serum-free α-MEM containing thymidine (10⁻³ M) with or without C₂-ceramide. After 48 h of culturing, the cells were fixed in 4% paraformaldehyde for 10 min, washed with 10 mM Tris–HCl, pH 8.0, and then permeabilized in 0.1% Triton X-100 in 10 mM Tris–HCl, pH 8.0, for 5 min. After washing with 10 mM Tris–HCl, pH 8.0, the cells were preincubated for 10 min at room temperature in the reaction buffer containing terminal deoxynucleotidyl transferase (200 mM potassium cacodylate, 0.22 mg/ml BSA and 25 mM Tris–HCl, pH 6–6). After 10 min, the preincubation buffer was removed and the reaction mixture containing 500 U/ml terminal deoxynucleotidyl transferase, 2.5 mM CoCl₂ and 40 μM biotinylated dUTP was added. After 60 min at 37 °C, the reaction was terminated by the addition of 300 mM NaCl and 30 mM sodium citrate. After 25 min at room temperature, cells were washed with PBS and incubated with streptavidin fluorescein for 60 min at room temperature in the dark. After extensive washing with PBS, the cells were examined using a Leica fluorescence microscope.

Statistical analysis

Data are presented as the mean ± s.e.m. of 6–12 cultures per group. Each experiment was repeated three times. Differences between the control and treatment groups were determined by the Mann–Whitney U test.

Results

The effect of exogenous cell-permeable C₂-ceramide on osteoblast viability was initially evaluated by culturing the cells in the absence or presence of increasing concentrations of C₂-ceramide followed by the determination of cell viability by the MTT assay. In the presence of 10% FCS, addition of C₂-ceramide has little effect on the viability of murine osteoblasts (data not shown). This is likely due to the strong interaction of C₂-ceramide with serum components that prevents its uptake (Spiegel & Merrill 1996). Cultures grown in 1% serum remained attached to the culture substratum, and viability was >80% for at least 24 h. Osteoblasts were exposed to various concentrations of exogenous ceramides for 24 h, and cell survival was measured using the MTT method. Murine osteoblasts demonstrated a dose-dependent increase in their survival rate when exposed to low concentrations of C₂-ceramide (5×10⁻⁹–10⁻⁷ M; Fig. 1). This effect was maximal (≈50%) in the presence of 5×10⁻⁸ M C₂-ceramide. However, increasing concentrations of C₂-ceramide (2×10⁻⁵–10⁻⁴ M) caused a dose-dependent decrease in mitochondrial succinate dehydrogenase activity and osteoblast survival compared with the control cultures (Fig. 1). In contrast, a similar dose of the inactive ceramide analogue, C₂-dihydroceramide, had no effect on osteoblast cell viability. Because C₂-dihydroceramide differs from C₂-ceramide only in that it lacks the 4,5-trans double bond, these studies demonstrate the specificity of C₂-ceramide. Furthermore, structurally relevant compounds such as oleic acid, N,N-dimethylsphingosine and sphingosine had no effect on osteoblast cell death (data not shown). These results indicate that cell death is not a non-specific effect of α-lipid analogues, and that the fatty acid-derived structure of ceramide is critical for this activity.

To determine whether C₂-ceramide has a similar effect on differentiating and matrix-mineralizing osteoblasts, the cells were cultured with AA (for induction of differentiation) or AA and β-glycerophosphate (matrix mineralization) as described in Materials and Methods, and were assessed using the MTT assay. We found that 5×10⁻⁸ M C₂-ceramide induced a level of survival in the matrix-mineralizing osteoblasts at 97.3±4.5% which was similar to that observed in the proliferating cells at 102.3±4.5%. The effect on differentiating osteoblasts was less pronounced at 89.3±3.9%, which was still significantly different from the effect observed for the untreated controls at 70.2±3.4% (P<0.01).

When 5×10⁻⁵ M C₂-ceramide was added to these osteoblast populations at different stages of maturity, we found that it induced almost complete cell death in both differentiated and matrix-mineralizing osteoblasts, with survival rates of 3.2±0.9 and 2.1±0.8% respectively, whilst in the proliferating cells, the survival rate was 1.1±0.7%.

![Figure 1 The effect of C₂-ceramide on the survival of primary mouse osteoblasts in vitro. Mouse osteoblasts were cultured as described in Materials and Methods in the presence of increasing concentrations of C₂-ceramide or C₂-dihydroceramide. After 24 h, cell survival was assessed by MTT assay. Values are expressed as percent survival of the vehicle-treated controls (given as 100%). Each point is the mean±s.e.m. of six wells. The experiment was repeated three times. *P<0.05; **P<0.01.](attachment:figure1.jpg)
The experiment was repeated three times. *

Either 5×10^-8 M or 5×10^-5 M of C2-ceramide for 12, 24, 36, 48, 60, 72, 84 and 96 h, and cell survival was assessed by MTT assay. Values are expressed as percent survival of the vehicle-treated controls (given as 100%). Each point is the mean ± S.E.M. of six wells. The experiment was repeated three times. *P<0.05; **P<0.01.

To evaluate the time-dependent effect of C2-ceramide on osteoblasts, the cells were treated with either 5×10^-8 or 5×10^-5 M of C2-ceramide. In the presence of 5×10^-8 M C2-ceramide, 73.4±5.4% of osteoblasts were viable after 48 h, whereas in the absence of C2-ceramide, only 29.5±3.6% of osteoblasts survived (Fig. 2). In contrast, 5×10^-5 M C2-ceramide induced complete cell death after 36 h, whereas only 53.6±6.3% of the cells were dead in the control cultures (Fig. 2).

Effect of SMase on osteoblast survival

Natural intracellular ceramide levels can be increased by treatment of cells with SMase, which causes membrane hydrolysis of sphingomyelin (Hannun 1994). Treatment of primary osteoblasts for 24 h with SMase resulted in a biphasic effect on osteoblast survival. At low concentrations of SMase (1–20 mU/ml), osteoblast survival rates were increased compared with the control cultures, whilst at increasing concentrations of SMase (50–200 mU/ml), there was a concentration-dependent suppression in cell survival (Fig. 3). Overall, these data show that endogenous ceramide has a biphasic effect on osteoblast survival with low concentrations enhancing survival and higher concentrations reducing the viability of osteoblasts in a time-dependent and concentration-dependent manner.

Phosphocholine, another product of SM hydrolysis by SMase, had no effect on osteoblast survival, demonstrating that ceramide is the principle biologically active catabolite of SM hydrolysis that mediates the effect on osteoblast survival (data not shown). Phospholipases A and C had no effect on osteoblast survival/death, excluding the possibility of a non-specific activity of phospholipases in this activity (data not shown).

Verification of apoptotic cell death

DNA ELISA and TUNEL analysis quantitatively and qualitatively confirmed the trends observed with the MTT assay. Initial experiments (data not shown) demonstrated 12 h to be the best treatment duration with C2-ceramide when quantifying apoptosis by DNA ELISA. C2-ceramide increased cytoplasmic histone-associated DNA fragments by 5.7- and 11.2-fold at 5×10^-7 and 10^-4 M C2-ceramide concentrations respectively in osteoblasts (Table 1). These results indicate that at these higher concentrations, C2-ceramide is a potent inducer of apoptosis in osteoblasts. Furthermore, SMase (200 U/ml) induced a 5.4-fold increase in cytoplasmic histone-associated DNA fragments in primary osteoblasts (Table 1).

Table 1 Effects of C2-ceramide and sphingomyelinase on osteoblast apoptosis. Mouse osteoblasts were cultured as described in Materials and Methods in the presence of either medium, C2-ceramide or SMase. After 12 h, apoptosis was assessed by Cell Death Detection ELISA as described in Materials and Methods. Values are expressed as DNA fragmentation with respect to the vehicle-treated controls (given as onefold). Each point is the mean ± S.E.M. of six wells. The experiment was repeated three times.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>DNA fragmentation/apoptosis index (S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>C2-ceramide</td>
<td>5×10^-5 M</td>
<td>5.7±0.6**</td>
</tr>
<tr>
<td>C2-ceramide</td>
<td>10^-4 M</td>
<td>11.2±1.0**</td>
</tr>
<tr>
<td>SMase</td>
<td>125 mU/ml</td>
<td>3.4±0.3**</td>
</tr>
<tr>
<td>SMase</td>
<td>200 mU/ml</td>
<td>7.2±0.6**</td>
</tr>
</tbody>
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**P<0.01.
Confirmation that the osteoblasts underwent apoptosis in the presence of C2-ceramide \( (5 \times 10^{-5} \text{ M}) \) or SMase \( (200 \text{ mU/ml}) \) was provided by the TUNEL technique (Fig. 4), with osteoblasts demonstrating clear cut staining indicative of chromatin condensation at the nuclear membrane.

Effect of \( \alpha \)-erythro-MAPP on osteoblast survival

To clarify further whether ceramide among sphingomyelin metabolites is responsible for promoting osteoblast survival, we examined the effect of \( \alpha \)-erythro-MAPP, an inhibitor of ceramidase, and therefore ceramide metabolism (Bielawska et al. 1996). \( \alpha \)-erythro-MAPP, which alone did not affect the basal level of osteoblast apoptosis, significantly amplified the effect of both ceramide \( (5 \times 10^{-5} \text{ M}) \) and SMase \( (125 \text{ mU/ml}) \) on osteoblast apoptosis (Fig. 5).

Glucosylation of ceramide is not required for C2-ceramide-induced survival or death

To study whether the conversion of C2-ceramide to C2-glucosylceramide is required for its survival- and death-promoting activities, threo-C2-ceramide, a stereoisomer of

![Figure 4](http://dx.doi.org/10.1677/JOE-10-0068)

*In situ* DNA labelling by the TUNEL technique. Mouse osteoblasts were cultured as described in Materials and Methods in the absence (A) or presence (B) of C2-ceramide \( (5 \times 10^{-5} \text{ M}) \) or (C) 125 mU/ml of sphingomyelinase. After 12 h, the cells were fixed, permeabilized and processed for TUNEL. (A) Typical example of osteoblasts showing negligible DNA fragmentation; (B and C) cells cultured in the presence of C2-ceramide or SMase showing extensive DNA fragmentation depicted by arrowheads. Bar = 10 µM. Full colour version of this figure available via http://dx.doi.org/10.1677/JOE-10-0068.

![Figure 5](http://dx.doi.org/10.1677/JOE-10-0068)

DMAPP enhances the apoptotic effect of C2-ceramide and SMase on primary mouse osteoblasts. Mouse osteoblasts were cultured as described in Materials and Methods in the presence of either medium, C2-ceramide \( (5 \times 10^{-5} \text{ M}) \), or SMase \( (125 \text{ mU/ml}) \) alone or in combination with DMAPP \( (7.5 \times 10^{-5} \text{ M}) \). After 24 h, apoptosis was assessed by Cell Death Detection ELISA as described in Materials and Methods. Values are expressed as DNA fragmentation with respect to the vehicle-treated controls (given as onefold). Each point is the mean ± S.E.M. of six wells. The experiment was repeated three times. *P < 0.05; **P < 0.01.
C2-ceramide, was added to the osteoblast cultures. Threeo-C2-ceramide is an analogue of ceramide that is not metabolized to threeo-C2-glucosylceramide (Pagano & Martin 1988). As in the case of C2-ceramide treatment, the viability of osteoblasts was dependent on the concentration of threeo-C2-ceramide, with a concentration of $5 \times 10^{-5}$ M promoting survival and a concentration of $5 \times 10^{-6}$ M inducing PCD (Fig. 6). Furthermore, treatment with $5 \times 10^{-5}$ M of N-butyldeoxygalactonojirimycin, an inhibitor of ceramide glucosyltransferase (Platt et al. 1994), had no effect on C2-ceramide-mediated osteoblast survival or death (Fig. 6).

**Intercellular mechanisms that mediate the effect of ceramide on osteoblast viability**

Previous studies have demonstrated the importance of various intracellular signalling factors including PKC in the survival of osteoblasts (Longo et al. 2004). Therefore, the effect of PKC inhibitors on ceramide-induced survival/death of osteoblasts was examined.

We initially investigated the involvement of PKC using the pan-specific PKC inhibitor chelerythrine chloride.

**Figure 7** (A) Effect of inhibitors of protein kinase C, protein phosphatases and PI3 kinases on ceramide-mediated osteoblast survival. Mouse osteoblasts were cultured as described in Materials and Methods in the presence of C2-ceramide ($10^{-8}$ M) with and without either chelerythrine chloride ($2 \times 10^{-7}$ M), rottlerin ($3 \times 10^{-5}$ M), PKC ζ pseudosubstrate ($10^{-5}$ M), okadaic acid ($2 \times 10^{-10}$ or $2 \times 10^{-8}$ M), LY294002 ($10^{-7}$ M) or wortmannin ($10^{-7}$ M). After 24 h, cell survival was assessed by MTT assay. Values are expressed as percent survival of the vehicle-treated controls (given as 100%). Each point is the mean ± S.E.M. of six wells. The experiment was repeated three times. *P<0.05, **P<0.01, ***P<0.001. (B) Effect of inhibitors of protein kinase C, protein phosphatases and PI3 kinases on ceramide-mediated osteoblast apoptosis. Mouse osteoblasts were cultured as described in Materials and Methods in the presence of C2-ceramide ($5 \times 10^{-5}$ M) with and without chelerythrine chloride ($2 \times 10^{-7}$ M), rottlerin ($3 \times 10^{-5}$ M), PKC ζ pseudosubstrate ($10^{-5}$ M), okadaic acid ($2 \times 10^{-10}$ or $2 \times 10^{-8}$ M), LY294002 ($10^{-7}$ M) or wortmannin ($10^{-7}$ M). After 24 h, apoptosis was assessed by Cell Death Detection ELISA as described in Materials and Methods. Values are expressed as survival of the vehicle-treated controls (given as onefold). Each point is the mean ± S.E.M. of six wells. The experiment was repeated three times.

A submicromolar concentration attenuated the osteoblast survival-promoting effects of $5 \times 10^{-8}$ M ceramide by 49.3% (Fig. 7A), and reduced the cell death-promoting effects of $5 \times 10^{-5}$ M ceramide by 33.3% (Fig. 7B). We next investigated the role of different PKC isoforms. The PKC δ inhibitor, rottlerin ($3 \times 10^{-5}$ M), diminished the cell survival-promoting effects of $5 \times 10^{-5}$ M ceramide by 33.3% (Fig. 7A), and reduced the cell death-promoting effects of $5 \times 10^{-5}$ M ceramide by 33.3% (Fig. 7B). We next investigated the role of different PKC isoforms. The PKC δ inhibitor, rottlerin ($3 \times 10^{-5}$ M), diminished the cell

**Figure 6** Is glucosylation required for ceramide to exert its effects on primary osteoblasts? Mouse osteoblasts were cultured as described in Materials and Methods in the presence of either threeo-C2-ceramide ($5 \times 10^{-8}$ or $5 \times 10^{-5}$ M) or C2-ceramide ($5 \times 10^{-8}$ or $5 \times 10^{-7}$ M) with and without N-butyldeoxygalactonojirimycin (NB-DGJ). After 12 h, apoptosis was assessed by Cell Death Detection ELISA as described in Materials and Methods. Values are expressed as survival of the vehicle-treated controls (given as onefold). Each point is the mean ± S.E.M. of six wells. The experiment was repeated three times. *P<0.05, **P<0.01.
death-promoting effects of $5 \times 10^{-5}$ M ceramide by 76.4\% (Fig. 7B), but it had no effect on the survival-promoting effects of $5 \times 10^{-8}$ M ceramide (Fig. 7A). Conversely, the cell-permeable PKC $\zeta$ pseudosubstrate ($10^{-5}$ M) strongly diminished the cell survival-promoting effects of $5 \times 10^{-8}$ M ceramide by 43.2\% (Fig. 7A), but it had no effect on $5 \times 10^{-6}$ M ceramide-mediated cell death (Fig. 7B). As a result of a lack of specific inhibitors, other PKC isoforms were not investigated in the study.

Ceramide-activated protein phosphatases, including PP1 and PP2a, are activated by ceramide, and they participate in many mammalian signalling pathways (Mumsby & Walter 1993).

To distinguish between PP1- and PP2a-type activities, okadaic acid was added at concentrations that inhibit both ($2 \times 10^{-8}$ M) or PP2a specifically ($2 \times 10^{-10}$ M). High-dose okadaic acid ($2 \times 10^{-8}$ M) enhanced $5 \times 10^{-8}$ M ceramide-induced osteoblast survival by 17.2\%, whilst okadaic acid ($2 \times 10^{-8}$ M) reduced $5 \times 10^{-5}$ M ceramide-induced osteoblast apoptosis by 51.2\%. Low-dose okadaic acid ($2 \times 10^{-10}$ M) had little or no inhibitory effect on either osteoblast survival or death, indicating that the predominant phosphatase activated by ceramide was PP1 (Fig. 7).

Because signalling through phosphatidylinositol-3 kinases (PI3K) has been implicated in the anti-apoptotic effects of many GFs (Grey et al. 2003), we examined the effect of the specific inhibitors of PI3K, LY294002 and wortmannin, on C2-ceramide-induced osteoblast survival and apoptosis. As shown in Fig. 7, neither LY294002 nor wortmannin had any effect on C2-ceramide-induced survival or apoptosis.

**Discussion**

The differing role of ceramide in physiology and pathology is underscored by the apparent contradictory results that have been reported with respect to the effects of ceramide on survival, differentiation and cell death (Goodman & Mattson 1996).

In this study, we have shown that the *in vitro* application of exogenous cell-permeable C2-ceramide, but not of its inactive analogue C2-dihydroceramide, has a biphasic effect on the survival of murine osteoblasts, with low doses promoting cell survival and high doses inducing apoptosis. These effects of C2-ceramide were apparent in proliferating, differentiating and matrix-mineralizing osteoblasts. In addition, treatment with SMase, which causes the formation of endogenous intracellular ceramide, also had a similar biphasic effect on mouse osteoblasts *in vitro*. Furthermore, prevention of the intracellular breakdown of ceramide, generated by SMase, enhanced its apoptotic activity on murine osteoblasts.

Reports regarding the role of ceramide in cell death or survival in the cells of skeletal origin have been inconsistent; both protective and destructive functions for ceramide have been suggested. Thus, low doses of ceramide ($<1.5 \times 10^{-7}$ M) in certain models have stimulatory effects on the synthesis of interleukin-6 and retinoic acid in osteoblasts (Takeshita et al. 2000). In contrast, administration of high doses of ceramide analogues ($>5 \times 10^{-7}$ M) induced osteoblast apoptosis, which is associated with transcription of NF-$\kappa$B or NFKB; (Kitajima et al. 1996). Moreover, intracellular ceramide levels were significantly increased in MC3T3-E1 cells subjected to apoptotic stimuli (Kitajima et al. 1996).

An intriguing observation from this study was that treatment of cultured osteoblasts with up to $10^{-6}$ M of C2-ceramide promoted cell survival, whereas higher doses ($>10^{-5}$ M) induced apoptosis which was similar to that observed in neuronal cells (Furuya et al. 1998). It is largely unknown how ceramide possesses multiple functions, although different metabolites of ceramide may lead to distinct effects. It has been suggested that the protection observed with lower concentrations of ceramide is attributable to its conversion to glucosylceramide (a protective glycolipid), whereas at higher concentrations it may not be ‘detoxified’ in this manner (Irie & Hirabayashi 1998).

However, this seems unlikely for cultured osteoblasts, since treatment with the inhibitor of ceramide glucosylation failed to prevent the survival-promoting activity of C2-ceramide. Furthermore, three-C2-ceramide, which is not metabolized to glucosylceramide, had an activity that was similar to that of C2-ceramide. These findings indicate that ceramide is responsible for the activity.

At low doses, ceramide could protect cells against death induced by exposure to a variety of stresses, such as the absence of certain GFs. In our system, osteoblasts were cultured in the absence of mineralized matrix, which is an abundant source of trophic factors. In support of this, it has been shown that cell death of cultured osteoblasts is rescued by IGF1 and 2, FGF and platelet-derived GF (Hill et al. 1997). In our culture system, the elevation of chromatin condensation in osteoblasts was prevented by low doses of C2-ceramide, suggesting that ceramide promotes osteoblast survival, in addition to these trophic factors.

Different strategies have been used to demonstrate the involvement of C2-ceramide in cell death. The addition of short-chain ceramides to cell cultures initially demonstrated (Haimovitz-Friedman et al. 1994) the involvement of ceramide in the apoptotic process, but it is not clear whether exogenous ceramide has the same cellular targets as the relevant endogenous species (Ghidoni et al. 1999). *In situ* generation of ceramide by bSMase appeared to be an alternative to the use of non-physiological ceramide species. However, bSMase probably generates ceramide in the external leaflet of the plasma membrane, which requires subsequent translocation in the membrane for the propagation of the apoptotic signal.

The last option involves pharmacological agents that alter ceramide metabolism. Ceramide elevation should occur by blocking either its glycosylation or its hydrolysis into sphingosine. Our results showing that the generation of ceramide from sphingomyelin by SMase or prevention of its
subsequent breakdown with d-erythro MAPP suggest that endogenously generated ceramide and exogenous ceramide act in a similar manner on osteoblasts.

One dilemma that often arises with the use of exogenously added agents is whether or not the concentrations used to induce cell death in cultures are relevant to the endogenous levels found under physiological or pathological conditions. This question is difficult to address, in part, because ceramide levels may fluctuate rapidly. Additionally, the requirements for intracellular ceramide may vary considerably. It has been suggested that neuronal cells have higher endogenous levels of ceramide than fibroblasts (Riboni et al. 1997).

Several stresses that induce apoptosis increase the cellular level of ceramide (Hannun 1996). It has been shown that the incorporated lipid concentrations of ceramide are 0·2 and 0·7 fmol per cell when 2·5 $\times$ $10^{-6}$ and $10^{-5}$ M of ceramide respectively are administered to neurone cultures. Interestingly, it was found that the lower concentration induced survival and the higher concentration induced apoptosis in the neurone cultures. This suggests that a critical point may exist between these two values, which is a point when the action of ceramide is directed towards cell survival or death.

One explanation is that the context of the ceramide signal determines the ultimate biological response to the molecule (Kolesnick & Fuks 1995). In this regard, ceramide-mediated apoptosis appears to be subject to transmodulatory control through 1,2-diacylglycerol (DAG)/protein kinase C (PKC); apoptosis initiated by ceramide analogues can be inhibited by an increase in DAG concentration (Kolesnick & Kronke 1998). Thus, even within the same cell type, an increase in ceramide concentrations may lead to different end points depending on the microenvironment in which the signal is generated.

From our results, it appears that the effects of ceramide on osteoblast survival and apoptosis may work through separate signal transduction routes. Whilst PKC $\zeta$ and the conventional PKCs appear to be involved in the mediation of the effects of ceramide on osteoblast survival, PP1 and PKC $\delta$ mediate the effects of ceramide on osteoblast apoptosis. There is evidence to support a role for both PKCs and PPs in ceramide-mediated cell signalling, albeit not in osteoblasts. Previous studies have shown that ceramide has a biphasic effect on PKC $\zeta$, with low doses activating the kinase and high doses causing inhibition (Müller et al. 1995). Furthermore, PKC $\zeta$ is activated by ceramide resulting in the coactivation of NF-κB and cell survival (Wang et al. 1999).

Our finding that PP1 is involved in ceramide-mediated osteoblast apoptosis is in agreement with studies that have shown that ceramide activation of PP1 alters splicing of BCL-x (BCL2L1 as listed in the MGI Database) and caspase 9 from anti- to pro-apoptotic proteins (Ghosh et al. 2007). Furthermore, ceramide activation of PP1 has been shown to inhibit the survival-promoting activity of PKC $\alpha$ (Lee et al. 1996). It is now clear that the PKC-dependent promotion or suppression of apoptosis occurs as a function of the isoform(s) that are activated and the cell type under investigation.

Genetic disruption of individual PKC isoforms in mice indicates that PKC $\alpha$, PKC $\beta$ and PKC $\zeta$ suppress apoptosis, whilst PKC $\delta$ is a critical proapoptotic signal in many cell types (Reyland 2009). Whilst we have demonstrated the involvement of PKCs and PP1 in ceramide-mediated apoptosis/survival, there was incomplete suppression of ceramide activity, suggesting that other mechanisms may be involved. Although there is evidence to suggest that caspases are involved in the ceramide signalling cascade (Hannun 1996), it has not been clearly determined whether ceramide acts up- or downstream to or independently of caspases.

Other pathways to consider

Gudz et al. (1997) reported direct inhibition of mitochondrial respiratory chain complex III by C2-ceramide, with half-maximal effect at 5–7 $\times$ $10^{-6}$ M in isolated mitochondria. However, at low concentrations 10$^{-6}$ M C2-ceramide protects some cells against toxic and oxidative insults (Goodman & Mattson 1996). Lipid peroxidation induced by these insults was significantly reduced by C2-ceramide (Goodman & Mattson 1996). Although the mechanism of this influence on mitochondrial reactions remains unclear, these effects might have played roles in our experiment and promoted survival at a low dose of C2-ceramide, whilst causing apoptosis at a high dose.

It has been reported that SMase is secreted from vascular endothelial cells, which cooperate with functional bone cells, osteoblasts and osteoclasts in bone remodelling (Erlebacher et al. 1995, Marathe et al. 1998). Taking our present findings into account, it is most likely that SMase plays an important role as an intercellular messenger in bone remodelling in cells that range from capillary endothelial cells to osteoblasts, stimulating ceramide synthesis, which may promote osteoblast survival.

In conclusion, the present results demonstrate that cell survival and death in murine osteoblasts can be induced by ceramide and SMase in a concentration-dependent manner. Furthermore, these effects are mediated by the activation of PKCs and PP1. These observations suggest that ceramide accumulation may be a generalized feature of osteoblast cell death. Further studies to identify whether the cell survival effects of ceramide influence the function and differentiation of osteoblasts and whether ceramide acts as a second messenger in the pro-survival effects of GFs such as IGF and cell death–promoting activity of TNF$\zeta$ may therefore improve our understanding of this lipid in osteoblast physiology.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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References


Erelbacher A, Filvaroff EH, Giteleman SE & Derynck R 1995 Toward a public, commercial or not-for-profit sector. This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.


Irie F & Hirabayashi Y 1998 Application of exogenous ceramide to cultured rat spinal motoneurons promotes survival or death by regulation of apoptosis depending on its concentrations. Journal of Neuroscience Research 54 475–485.


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