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Abstract

The underlying mechanisms that allow proinflammatory cytokines to exert a direct adverse effect on growth plate chondrogenesis are unclear. These effects may be mediated by ceramide, a sphingosine-based lipid second messenger, which is elevated in a number of chronic inflammatory diseases. To test this hypothesis, we determined the effects of C2-ceramide a cell permeable ceramide analogue, on the growth of the ATDC5 chondrogenic cell line and on cultured fetal mice metatarsals.

In ATDC5 cells, C2-ceramide significantly induced apoptosis at both 40μM (82%; $P<0.05$) and 25μM (53%; $P<0.05$). At 40μM, C2-ceramide significantly reduced proliferation ($[^3H]$-thymidine uptake/mg protein) (62%; $P<0.05$). Cell number was significantly reduced at both 40μM (72%; $P<0.05$) and 25μM (62%; $P<0.05$). C2-ceramide did not markedly alter the mRNA expression of markers of chondrogenesis (sox 9 and collagen II) and differentiation (aggrecan and collagen X). Both, in the presence and absence of IGF-1, C2-ceramide (25μM) induced an equivalent amount of reduction (60%) in proliferation ($P<0.001$). Similarly, C2-ceramide (40μM) induced a 31% reduction in fetal metatarsal growth both in the presence and absence of IGF-1 (both $P<0.001$). Therefore, C2-ceramide does not appear to specifically inhibit the pro-proliferative effects of exogenous IGF-1. AG1024, an IGF-1 and insulin receptor blocker, reduced proliferation by 28% compared to control cells ($P<0.001$). C2-ceramide (25μM) significantly reduced proliferation compared to AG1024 treatment (55%, $P<0.001$). C2-ceramide and AG1024 in combination further reduced proliferation compared to C2-ceramide alone (46 %; $P<0.01$). Cell signalling studies demonstrated that C2-ceramide (25μM) treatment for 24h did not alter IGF-1-stimulated phosphorylation of IRS-1, Akt or Erk 1/2.

In conclusion, C2-ceramide inhibits proliferation and induces apoptosis in growth plate chondrocytes through an IGF-1 independent mechanism.

Introduction

Chronic inflammatory diseases such as Juvenile Idiopathic Arthritis (JIA) and Inflammatory Bowel Disease (IBD) often lead to childhood growth retardation through a number of proposed mechanisms that includes nutritional deficiency, chronic inflammation, increased catabolism, defects in the GH/IGF-1 axis and use of glucocorticoids (MacRae et al. 2006a; Wong et al. 2006). Proinflammatory cytokines such as TNFα and IL-1β, are often raised in these diseases, and measures that block TNFα action such as anti-TNF therapy lead to an improvement in growth, an effect which has been reported to be independent of the concurrent reduction in the need for therapy with glucocorticoids (Tynjala et al. 2006). TNFα and IL-1β, may directly inhibit growth plate chondrocyte dynamics as well as longitudinal growth *in vitro* Martensson et al. 2004; MacRae et al. 2006b) but the underlying mechanisms that lead to these effects are unclear.

The insulin-like growth factor–I (IGF-I) signalling pathway is the major autocrine/paracrine regulator of bone growth (Loveridge et al. 1990). Binding of IGF-I to its receptor utilises a family of soluble receptors, known as insulin receptor substrates (IRSs), to initiate a series of autophosphorylation events. This results in the activation of two distinct signalling pathways, phosphatidylinositol 3-kinase (PI-3K) and p44/p42 mitogen-activated protein kinase (MAPK), leading to pro-proliferative and anti-apoptotic effects.

In many cell types, signal transduction of IL-1β and TNFα involves the activation of neutral (N) and acidic (A) sphingomyelinase (Smase) pathways, which catalyse the

degradation of the membrane phospholipid sphingomyelin into phosphocholine and ceramide (Mathias et al. 1993; Weigmann et al. 1994; Rybakina et al. 2001). Ceramide is also elevated through a de novo synthesis pathway following activation of IL-1β and TNFα receptors (Memon et al. 1998; Xu et al. 1998). Ceramide has been shown to inhibit IGF-1-induced tyrosine phosphorylation of IRS-1 in myoblast and hepatic cells (Kanety et al. 1996; Strle et al. 2004). Ceramide has also been shown to induce apoptosis in a wide range of different cell types, including pancreatic beta cells (Sjoholm et al. 2005), cardiomyocytes (de Vries et al.1997), astrocytes (Oh et al. 2006) and articular chondrocytes (Sabatini et al. 2000).

Whilst previous studies that have shown that IL-1β and TNFα inhibit growth plate chondrocyte differentiation and induce cell death (Martensson et al. 2004; MacRae et al. 2006b), the direct effects of ceramide on growth plate chondrocytes have yet to be reported. In this study, the ATDC5 chondrogenic cell line was used to characterise and compare the effects of ceramide on cell proliferation, differentiation and apoptosis. Subsequently, the effect of ceramide on IGF-1 signalling was examined using ATDC5 cells and the fetal murine metatarsal model.

Materials & Methods

Chondrocyte cell culture

The ATDC5 chondrocyte cell line was sourced from the RIKEN cell bank (Ibaraki, Japan) and maintained as described by Atsumi et al. (1990). Cells were maintained in T175 tissue-culture flasks (Greiner Bio-One GmbH, Frickenhausen, Baden-Württemberg, Germany) at a density of 250,000 cells/flask in a maintenance medium of DMEM/Ham's F12 (Invitrogen, Paisley, Strathclyde, UK) supplemented with 5% FCS (Invitrogen), $10\mu gml^{-1}$ human transferrin, $3 \times 10^{-8}M$ sodium selenite (Sigma, Poole, Dorset, UK), sodium pyruvate (1mM; Invitrogen) and gentamycin ($50\mu g/ml$ Invitrogen). For individual experiments semi-confluent cultures were passaged with trypsin-EDTA (Sigma) and cultured (Day 0) at a density of $6,000$ cells/cm^2 in multi-well plates (Costar, High Wycombe, Bucks, UK) in a differentiation medium that consisted of maintenance medium supplemented with insulin ($10\mu g/ml$; Sigma). Incubation was at 37°C in a humidified atmosphere of 95% air / 5% CO_2 and the medium was changed every second / third day. In all experiments, unless otherwise stated, C2-ceramide (Sigma), a cell permeable ceramide analogue, was added to chondrocyte cultures on Day 6, at a final concentration of 40, 25 and $10\mu M$. The diluent for C2-ceramide was ethanol (final concentration 0.1%). All control cultures received 0.1% ethanol only.

ATDC5 cells were deprived of serum and ITS for 18h before the initiation of treatments in the presence of IGF-1 (100ng/ml) (Bachem (UK) Ltd., St. Helens, Merseyside, UK), and before the initiation of the cell signalling studies.

Inhibition of ceramide generation following pro-inflammatory cytokine exposure.

D609 (10μg/ml), an inhibitor of A-SMase activity, was added to cells cultured in 48 well plates, in the presence of IL-1β and TNFα (both 10ng/ml). The rate of chondrocyte proliferation was assessed over a 24 hour period starting on Day 6. On Day 7, the chondrocytes were incubated with 0.2μCi/ml [³H]thymidine (37MBq/ml; Amersham Pharmacia Biotech, Bucks, UK) for the last 2 hours of the culture period. The amount of radioactivity incorporated into trichloroacetic acid-insoluble precipitates was measured (Farquharson et al. 1999).

Chondrocyte number, proliferation and apoptosis

Protein content was determined as a measure of cell number. Cells were cultured in 12 well plates, and exposed to C2-ceramide on Day 6 and Day 8. On Day 10, the cells were washed twice with phosphate buffered saline, lysed with 0.9% NaCl and 0.2% Triton X-100 and centrifuged at 12000*g* for 15min at 4°C. The protein content of the supernatant was measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories) based on the Bradford dye binding procedure, and gamma globulin as standard (Farquharson et al. 1995). Proliferation was determined as described above. Apoptosis of the cells was measured using the APO Percentage Apoptosis assay, (Biocolor Ltd, Belfast, N Ireland), which quantifies dye uptake by apoptotic cells only after the translocation of phosphatidylserine to the outer surface of the cell membrane (Fadok et al. 1992). Apoptosis was assessed following the manufacturer's protocol in cells cultured in 48 well plates over a 24-hour period starting on Day 6.

Analysis of chondrogenic gene expression

On days 13 and 15, C2-ceramide was added to cells cultured in 6 well plates. During this period the cells express established markers of the chondrocyte differentiated phenotype (Mushtaq et al. 2002). The experiment was stopped on day 17 and total RNA was extracted from chondrocytes by repeated aspiration through a 25-gauge syringe needle in 0.5ml Ultraspec (Biotecx, Houston, TX, USA). Following extraction with chloroform, RNA in the aqueous phase was precipitated with isopropanol and bound to RNA Tack resin (Biotecx) following the manufacturer's protocol. After washing with 75% ethanol, the RNA was eluted in 100μl ribonuclease –free water (Houston et al. 1999). For each sample, total RNA content was assessed by absorbance at 260nm and purity by A260/A280 ratios, which were 1.9-2.0 in all cases. All preparations were diluted to a concentration of 50ng/μl and stored at -70°C. Gene expression was analysed by semi-quantitative RT-PCR (Jefferies et al. 1998; Farquharson et al. 1999; Houston et al. 1999; Jefferies et al. 2000). Aliquots of 500ng RNA (or an equivalent volume of water as a control) were reverse transcribed in 20μl reactions containing cDNA equivalent to 10ng RNA and 200nM gene-specific primers in 11.1 X PCR buffer (Jefferies et al. 2000). The cycling profile was 1 min at 92°C (first cycle, 2 min), 1 min at 55°C, and 1 min at 70°C. The number of cycles performed was carefully titrated to ensure that the reactions were in the exponential phase. Reaction products were analysed on 1.5% agarose gels in the presence of ethidium bromide (250μg/l), and a digital image of each gel was captured using a gel documentation system (Bio-Rad Laboratories, Inc., Hemel Hempstead, Herts, UK).

IGF-1 studies in ATDC5 cells

The cells were treated with C2-ceramide (25μM) for 24h in the presence of IGF-1. Proliferation was determined as described above. In further studies, the cells were treated with C2-ceramide (25μM) in the presence of IGF-1 in the presence of AG1024, an IGF-1 and insulin receptor blocker, (10μM) (Sigma) for 24h (Lee et al. 2005; Sutter et al. 2006). The diluent for AG1024 was dimethylsulfoxide (final concentration 0.1%). Control cultures received 0.1% dimethylsulfoxide only. Proliferation was determined as described above. The minimum concentration at which ceramide could inhibit IGF-1-induced proliferation in ATDC5 cells was examined using C2-ceramide concentrations between 10μM and 40μM at 5μM intervals using serum free medium.

Western blotting analysis

ATDC5 cells were treated with C2-ceramide (25μM) for 24h, and then either lysed immediately or stimulated with IGF-1 (100ng/ml) for 10 min and then lysed. Cells were lysed in PhosphoSafe extraction buffer (Merck Biosciences Ltd, Nottingham, UK) containing "Complete" protease inhibitor cocktail (Roche, East Sussex, UK) according to manufacterer's instructions. Lysates (corresponding to 25μg protein) were run on 10% Bis-Tris gels (Invitrogen, Paisley, UK) and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline/Tween-20 (TBST; 50mM Tris-HCl, 300mM NaCl, 0.1% Tween-20, pH 7.6) at 4°C overnight with gentle shaking. The membranes were probed for 1 hour at room temperature with primary antibodies (all 1:1000 dilution in 5% milk) against phospho-Akt (ser 473), total Akt, phospho-P44/42 Map Kinase (Thr202-/Tyr204), total P44/42 Map Kinase, Phospho-SAPK/JNK (Thr183/Tyr185), total

SAPK/JNK, Phospho-p38 MAP Kinase (Thr180/Tyr182) and total p38 MAP Kinase (Cell Signaling Technology, Beverly, MA). The blots were washed 4×15 min in TBST. The membranes were then incubated with anti-rabbit IgG-peroxidase (Cell Signalling Technology) for 1h (1:1000 dilution in 5% milk). The membranes were washed, as described above, and developed using the ECL-plus Western Blotting Detection System (Amersham Biosciences, Buckinghamshire, UK).

Immunoprecipitation of IRS-1

ATDC5 cells were treated with C2-ceramide (25μM) for 24h, and then stimulated with IGF-1 (100ng/ml) for 10 min. Cells were then lysed in lysis buffer (150mM NaCl ; 10mM Tris.HCl pH 7.4 ; 0.5 % NP40 ; 1mM Sodium Vanadate; 0.5M EDTA; 0.1M PMSF; 4mg/l aprotinin. IRS-1 was immunoprecipitated by incubating cell lysates overnight at 4°C with 2 μg/ml of anti - IRS-1 antibody and 30μl of protein A-Sepharose beads. Protein bound to the beads was washed four times with lysis buffer, and 10ul aliquots were run on 3-8% Tris-Acetate gels (Invitrogen) and transferred onto a nitrocellulose membrane. The membranes were blocked as described above, and then probed for 1 hour at room temperature with antiphosphotyrosine clone 4G10 and total IRS-1 antibodies (Upstate, Lake Placid, New York, USA). The blots were washed 4×15 min in TBST. The membranes were then incubated with anti-mouse IgG-peroxidase (Cell Signalling Technology) for 1h (1:1000 dilution in 5% milk). The membranes were washed and developed as described above.

Organ culture

The middle three metatarsals were aseptically dissected from 19-day-old embryonic Swiss mice. Bones were cultured at 37°C in a humidified atmosphere of 95% air / 5% CO_2 in 24 well plates. Each culture well contained 300μl of αMEM (Invitrogen) supplemented with 0.2% Bovine Serum Albumin, Fraction V (Sigma); 1mmol/l β-glycerophosphate (Sigma); 0.05mg/ml L-ascorbic acid phosphate (Wako Pure Chemicals Ltd, Neuss, North Rhine-Westphalia, Germany); 0.05mg/ml gentamycin and 1.25ug/ml fungizone (Invitrogen) (Mushtaq et al. 2004). C2-ceramide was added at a final concentration of 40, 30, 20 and 10μM for an 8 day period. In further studies with IGF-1, C2-ceramide was added at a final concentration of 40μM for an 8 day period, in the presence of IGF-1 (100ng/ml). The minimum concentration at which ceramide could affect metatarsal growth was examined using C2-ceramide concentrations between 0μM and 40μM at 10μM intervals using serum free medium in the fetal metatarsal culture model. In all experiments, the medium was changed every second/third day.

Morphometric analysis

Digital images of the metatarsals were captured every second day of culture and viewed on a Nikon Eclipse TE3000 microscope (Nikon, Kingston upon Thames Surrey, UK), using a digital camera (DS Camera Head DS-5M; Nikon). The total length of the bone through the centre of the mineralising zone was determined using image analysis software (DS Camera Control Unit DS-L1; Nikon). All results are expressed as a percentage change from harvesting length, which was regarded as baseline.

Statistical analysis

All experiments were performed at least twice. General Linear Model analysis was used to assess the data. All data are expressed as the mean +/- S.E.M. of six observations within each experiment. Statistical analysis was performed using Minitab 14. *P*<0.05 was considered to be significant.

Results

Ceramide generation following pro-inflammatory cytokine exposure.

TNFα (10ng/ml) significantly reduced proliferation compared to control cells (Fig. 1; 93%; P<0.001). When added in the presence of TNFα, D609 (10μg/ml) significantly increased proliferation compared to cytokine treatment alone (Fig. 1; 927%; P<0.001). D609 also significantly increased proliferation in control cells (Fig. 1; 84%; P<0.001).

Characterisation of the effect of C2-ceramide on ATDC5 cells.

In the ATDC5 cells, 40μM C2-ceramide significantly reduced cell proliferation over a 24h period (Fig. 2a), and this was confirmed when corrected for protein content (Fig. 2b). Following a 5 day exposure period, both 40 and 25μM C2-ceramide significantly reduced cell number (protein content) (Fig. 2c). Apoptosis was also increased at both concentrations following exposure for 24h (Fig. 2d). There was no significant alteration in C2-ceramide-induced mRNA expression of markers of chondrogenesis or differentiation (sox 9, collagen II aggrecan and collagen X) at all concentrations studied (Fig. 3).

IGF-1 studies

The lowest concentration of C2-ceramide that inhibited ATDC5 cell proliferation following 18h serum deprivation was 25μM in the presence of IGF-1 (Fig. 4a). Cells exposed to C2-ceramide (25μM) in the presence or absence of IGF-1 (100ng/ml) for 24h indicated that IGF-1 alone significantly increased proliferation (Fig 4b; $P<0.001$). In the presence of IGF-1, C2-ceramide induced a 68% reduction in proliferation (Fig. 4b; $P<0.001$). However, in the absence of IGF-1, ceramide induced a comparable 61% decrease (Fig. 4b; $P<0.001$).

In the more physiological fetal metatarsal model, the lowest concentration of C2-ceramide that significantly reduced fetal metatarsal growth was 40μM in the presence of IGF-1 (Fig. 5a; 62%; $P<0.05$). C2-ceramide (40μM) induced the same 31% reduction in metatarsal growth both in the presence and absence of IGF-1 (9d, 100ng/ml) (Fig. 5b; both $P<0.001$).

The effect of C2-ceramide on endogenous IGF-1 induced ATDC5 cell proliferation was examined, using AG1024, an IGF-1 and insulin receptor blocker (10μM) (Fig. 4c). In the absence of exogenous IGF-1, AG1024, an IGF-1 and insulin receptor blocker, reduced proliferation by 28% compared to control cells ($P<0.001$). C2-ceramide (25μM) significantly reduced proliferation compared to AG1024 treatment (55%, $P<0.001$). C2-ceramide and AG1024 in combination further reduced proliferation compared to C2-ceramide alone (46 %; $P<0.01$).

Cell signalling

24h C2-ceramide exposure did not inhibit the IGF-1 induced phosphorylation of insulin receptor substrate −1 (IRS-1), Akt (protein kinase B) or P44/42 Map Kinase (Erk 1/2) (Fig. 6). Further studies revealed that 24h C2-ceramide exposure did not alter Phospho-SAPK/JNK, Phospho-p38 MAP Kinase or phospho-P44/42 Map Kinase (Erk 1/2) activity (data not shown).

Discussion

Ceramide is an intracellular second messenger, whose signalling plays an important role in the regulation of cell proliferation, differentiation and survival (Kolesnick 2002; Ruvolo 2002; Menaldino 2003). This is the first study to demonstrate that C2-ceramide inhibits proliferation and induces apoptosis in growth plate chondrocytes. Ceramide has been shown to induce apoptosis in a wide range of different cell types, including pancreatic beta cells (Sjoholm et al. 2005), cardiomyocytes (de Vries et al. 1997), astrocytes (Oh et al. 2006) and articular chondrocytes (Sabatini et al. 2000).

An increase in ATDC5 proliferation was observed with TNFα exposure in the presence of D609, an inhibitor of ceramide generation. This suggests that ceramide may mediate the effects of pro-inflammatory cytokines in growth plate chondrocytes. An increase in proliferation was also observed in presence of D609 in the control cells, demonstrating that growth plate chondrocytes have an endogenous production of ceramide. Blocking this endogenous production therefore acts as an internal lid on proliferation.

Standardisation of [^{3}H] thymidine uptake with protein content confirms that an actual reduction in proliferation was observed following ceramide exposure, rather than solely a reduced uptake due in increased apoptosis and therefore fewer cells. Both

TNFα and IL-1β exposure have also been reported to inhibit proliferation and induce apoptosis in ATDC5 cells (MacRae et al. 2006b), suggesting that ceramide may be acting as a second messenger. Both IL-1B and TNFa have been shown to markedly reduce the mRNA expression of collagen II, collagen X and aggrecan (MacRae et al. 2006b) but in our current study ceramide did not alter the mRNA expression of markers of chondrogenesis and differentiation (sox 9, collagen II, aggrecan and collagen X) suggesting that ceramide generation may be just one of many different pathways through which the pro-inflammatory cytokines act.

Having established that C2-ceramide inhibits proliferation, and induces apoptosis, we went on to investigate whether these effects are mediated through inhibition of the IGF-1 signalling pathway, which is the major autocrine/paracrine regulator of bone growth (Loveridge et al. 1990). Strle et al. (2004) demonstrated that ceramide inhibits IGF-1 induced protein synthesis and differentiation in myoblasts. However, it has also reported that ceramide can inhibit myoblast growth and differentiation in the absence of exogenous IGF-1 (Meadows et al. 2000; Strle et al. 2004). It has been proposed that the discrepancies between these results can be attributed to the use of excessively high concentrations of C2-ceramide, and using cell culture media supplemented with low concentrations of serum (Strle et al. 2004). In our hands, the lowest concentration of C2-ceramide that inhibited ATDC5 cell proliferation following 18h serum deprivation was 25μM in the presence of IGF-1, compared to 40μM under standard culture conditions. This indicates that the ATDC5 cells are more susceptible to the actions of ceramide when serum deprived, and under stress.

C2-ceramide induced a comparable reduction in ATDC5 proliferation and fetal metatarsal growth both in the absence and presence of exogenous IGF-1. Furthermore, In the presence of AG1024, an IGF-1 and insulin receptor blocker, ceramide was still able to inhibit proliferation.

Cell signalling studies confirmed that ceramide did not inhibit the IGF-1 induced phosphorylation of IRS-1, or the major downstream pathways of IRS-1 - P44/42 Map Kinase and PI3 Kinase (as determined by Akt phosphorylation). Previous studies have reported that ceramide inhibits IGF-1 induced IRS-1 phosphorylation in certain cell types including myoblasts and hepatic cells (Kanety et al. 1996; Strle et al. 2004), but not in others, such as adipocytes (Summers et al. 1998) and motor neuron cells (Zhou et al. 1998). Ceramide has been shown to inhibit IGF-1 induced Akt phosporlyation in other cell types, including kidney cells, fibroblasts and adipocytes (Meier et al. 1998; Chen et al. 1999).

Involvement of MAPK subfamilies (P44/42 Map Kinase, c-Jun N-terminal kinase (JNK), and p38 kinase) in ceramide-induced apoptosis have been reported in various cell types including astrocytes (Blazquez et al. 2000; Oh et al. 2006), neuronal cells (Verheij et al. 1996; Willaime et al. 2001; Willaime-Morawek et al. 2003), lung cancer derived cells (Kurinna et al. 2004), vascular smooth muscle cells (Loidl et al. 2004) . The P44/42 Map Kinase pathway plays a major role in regulating cell growth, survival, and differentiation. In contrast, JNK and p38 pathways are activated in response to chemical and environmental stress (Xia et al. 1995; Cobb 1999). However, our studies showed that in growth plate chondrocytes, whilst ceramide induced apoptosis, the phosphorylation of P44/42 Map Kinase, JNK and p38 kinase was not altered.

Our previous studies have shown that IGF-1 plays a major role in promoting longitudinal growth in the mouse metatarsal model (Mushtaq et al. 2004). Furthermore the growth inhibitory effect of Dexamethasone was reversed in this model following exposure to IGF-1 (Mushtaq et al. 2004). However, the growth inhibitory effects of TNFa and IL1 are only partially reversed following treatment with IGF-1 (Martensson et al. 2004), suggesting that some of the effects of these proinflammatory cytokines may be IGF-1 independent. Furthermore, recombinant human growth hormone therapy in children with growth retardation and chronic inflammatory disease may result in a rise in IGF-1 and a cessation in further deterioration in growth (Davies et al. 1997; Bechtold et al. 2003). However, the failure to observe a clear normalisation of growth may be explained by the possibility that the growth retardation in these children may be due to a combination of a defect in growth regulatory pathways that are IGF-1 dependent and independent.

In conclusion, we have reported for the first time in growth plate chondrocytes, a link between pro-inflammatory cytokines and ceramide generation, and an anti-proliferative and pro-apoptotic effect of ceramide. Interestingly, these effects appear to be IGF-1 independent. Inflammatory cytokine induced generation of ceramide, and its subsequent actions on the growth plate, may be associated with the abnormal growth observed in children suffering from chronic inflammatory diseases.

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