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# Modulation of cell growth and differentiation by ceramide

Alicja Bielawska, Corinne M. Linardic and Yusuf A. Hannun

*Departments of Medicine and Cell Biology, Duke University Medical Center, Durham, NC 27710, USA*

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Ceramide has been suggested as an intracellular modulator of cell growth and differentiation [Okazaki, T. et al. (1990) J. Biol. Chem. 265, 15823–15831]. In this study, parameters that modulate the effects of ceramide on HL-60 cell growth and differentiation were examined. A short-chain, cell-permeable analog of ceramide, $C_2$-ceramide, induced differentiation of HL-60 human leukemia cells and inhibited HL-60 growth in a concentration-dependent manner. The potency of $C_2$-ceramide was modulated by the starting cell density such that the concentration of $C_2$-ceramide producing 50% inhibition of cell growth ($IC_{50\%}$) ranged from 2 $\mu$M (for cells suspended at $1 \times 10^5$ cells/ml) to 11 $\mu$M (for cells at $8 \times 10^5$ cells/ml). However, the $IC_{50\%}$ showed little variation if the concentration of $C_2$-ceramide was expressed as fmol of $C_2$-ceramide per $10^5$ cells. Therefore, the effectiveness of $C_2$-ceramide appeared to be primarily determined by its *cellular* rather than *molar* concentration. Binding of $C_2$-ceramide to serum proteins resulted in a 10-fold increase in the $IC_{50\%}$. These results demonstrate that the biologic activity of $C_2$-ceramide is subject to surface dilution kinetics and is sensitive to the presence of lipid-binding proteins. In these properties, ceramide behaves as a prototypic lipid second messenger/intracellular mediator.

Ceramide; Cell growth; Cell differentiation; Signal transduction; Sphingolipid

## 1. INTRODUCTION

Recent studies have led to the identification of a sphingomyelin cycle, whereby the action of extracellular agents such as 1,25-dihydroxyvitamin $D_3$ [1], tumor necrosis factor-$\alpha$ and $\gamma$-interferon [2] results in activation of a neutral sphingomyelinase. This causes the hydrolysis of membrane sphingomyelin and the generation of intracellular ceramide. Studies with bacterial sphingomyelinase and with cell-permeable analogs of ceramide suggest an important role for ceramide in the regulation of cell proliferation and differentiation [1–3]. Thus, short-chain ceramides were shown to inhibit HL-60 cell proliferation and to induce monocytic differentiation of these leukemia cells [2,3]. Although these short-chain ceramide analogs, such as $C_2$-ceramide (*N*-acetylsphingosine), have significantly more water solubility than natural ceramides, they nevertheless exhibit special properties as pharmacologic agents. They are amphiphilic molecules that are presumably active in the context of cell membranes (since they mimic the action of ceramide, an endogenous membrane lipid).

In our studies with $C_2$-ceramide, we found significant inter-experiment variability in the potency of ceramide; however, within an individual experiment, ceramide demonstrated very consistent effects (for example see Fig. 1). This variability was reminiscent of studies with sphingosine, another biologically active amphiphilic sphingolipid. In the case of sphingosine, biologic activity was modulated by the *ratio* of sphingosine to cell number [4] and by the presence or absence of lipid-binding proteins (such as bovine serum albumin or fetal calf serum [4,5]). Therefore, we examined the effects of these parameters on the action of $C_2$-ceramide.

*Correspondence address:* Y.A. Hannun, Departments of Medicine and Cell Biology, Box 3355, DUMC, Durham, NC 27710, USA. Fax: (1) (919) 681-8253.

## 2. EXPERIMENTAL

### 2.1. *$C_2$-ceramide*

$C_2$-ceramide was synthesized as previously described [3].

### 2.2. *HL-60 cell proliferation and differentiation*

HL-60 human myelocytic leukemia cells were purchased from ATCC and used between passages 20 and 45. The cells were grown in RPMI 1640 medium containing 10% fetal calf serum at 37°C in a 5% $CO_2$ incubator at densities ranging between $1 \times 10^5$ cells/ml to $1 \times 10^6$ cells/ml. The cells were washed once with phosphate-buffered saline and resuspended in serum-free media containing insulin (5 mg/l) and transferrin (5 mg/l) for 2–3 h. Cells were then treated with $C_2$-ceramide or with ethanol vehicle (ethanol concentration was less than 0.1%). For all reported experiments, cell viability was greater than 80%. For some experiments (Fig. 3), $C_2$-ceramide was delivered in a 1:1 complex with bovine serum albumin, as indicated in the relevant figure legend. Cells were counted using a hemocytometer, and cell viability was evaluated by Trypan blue dye exclusion. HL-60 cell differentiation was analyzed by measuring $H_2O_2$ production in a micro assay as described [6,7].

## 3. RESULTS

In serum-free media, $C_2$-ceramide was a potent inhibitor of HL-60 cell proliferation (Fig. 1A). In the experiment shown in Fig. 1A, $C_2$-ceramide at 2.5 $\mu$M produced near total inhibition of HL-60 cell proliferation

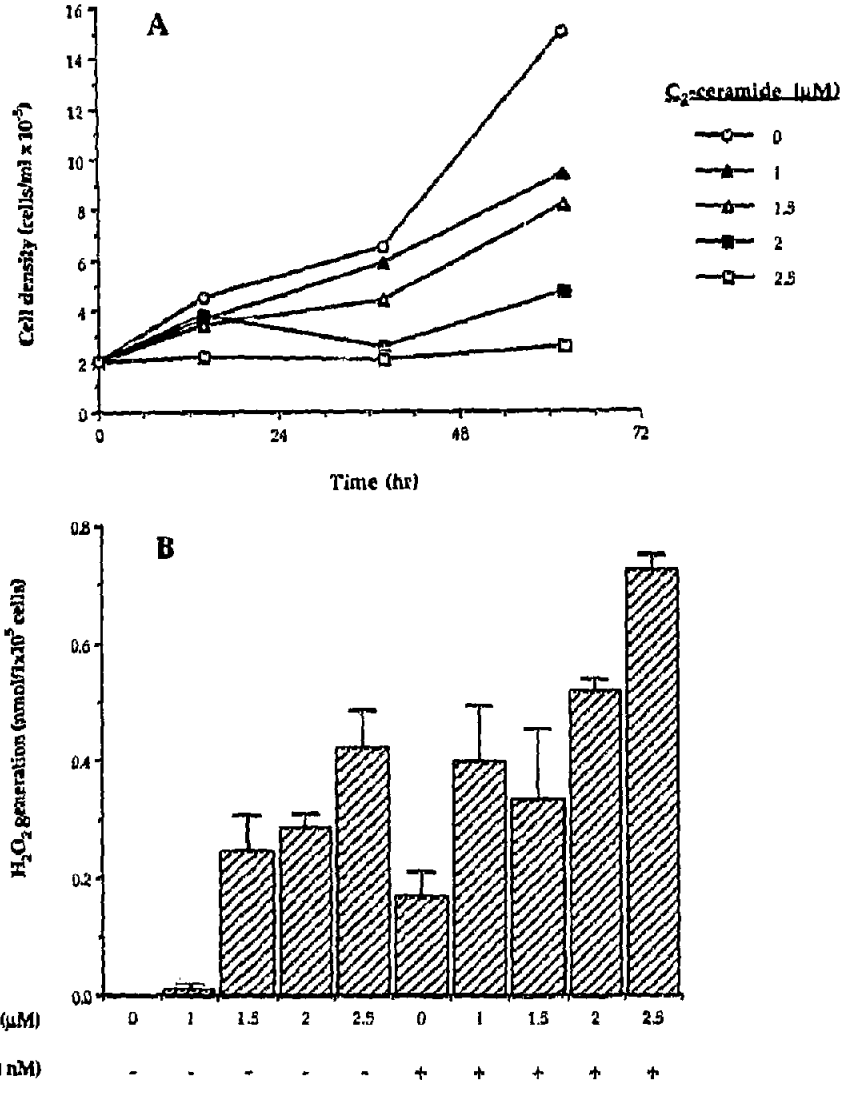


Fig. 1. Effects of $C_2$-ceramide on HL-60 growth and differentiation. (A) Time and concentration dependence of HL-60 growth on $C_2$-ceramide. HL-60 cells in serum-free media were treated with the indicated concentrations of $C_2$-ceramide and cell number was determined at the indicated time points. These results are representative of more than 10 experiments. (B) Effects of $C_2$-ceramide on $H_2O_2$ generation. HL-60 cells were treated with or without 1,25-dihydroxyvitamin $D_3$ (0.3 nM) in the presence of the indicated concentrations of $C_2$-ceramide. Differentiation was monitored by the ability of differentiated HL-60 cells to generate $H_2O_2$ following 3 days of treatment with $C_2$-ceramide. These results are averages of 2 determinations and are representative of 6 experiments.

without significant cell death. Higher concentrations resulted in increasing cytotoxic effects (data not shown). The precise concentration of $C_2$-ceramide required to produce growth stasis (e.g. 2.5 $\mu$M in Fig. 1) varied from experiment to experiment but was always less than cytotoxic concentrations (e.g. >5–10 $\mu$M).

$C_2$-ceramide alone or in combination with low concentrations of 1,25-dihydroxyvitamin $D_3$ resulted in significant induction of HL-60 cell differentiation as judged by the ability to induce NBT-reducing activity [1,3] and the ability to induce $H_2O_2$ production (Fig. 1B). The dose response for induction of $H_2O_2$ resembled that required for inhibition of cell growth with maximal stimulation of differentiation ($H_2O_2$ production) occurring in response to 2.5 $\mu$M of $C_2$-ceramide. The ability of $C_2$-ceramide to induce differentiation suggests that ceramide is activating a pathway that is involved in regulation of cell proliferation and differentiation and that the effects on cell proliferation are unlikely to be due to non-specific action of ceramide.

We next addressed the question of whether starting cell density was an important parameter in modulating ceramide potency. HL-60 cells at different starting cell densities were treated with variable concentrations of $C_2$-ceramide, and proliferation was monitored (Fig. 2). $C_2$-ceramide was able to inhibit HL-60 cell growth in a dose-dependent manner at all starting cell densities (Fig. 2A). However, it became obvious that the potency of ceramide was modulated significantly by the starting cell density. For example, $C_2$-ceramide was much more potent in inhibiting HL-60 cell growth at the lower starting cell density of $1 \times 10^5$ cells/ml than at higher cell

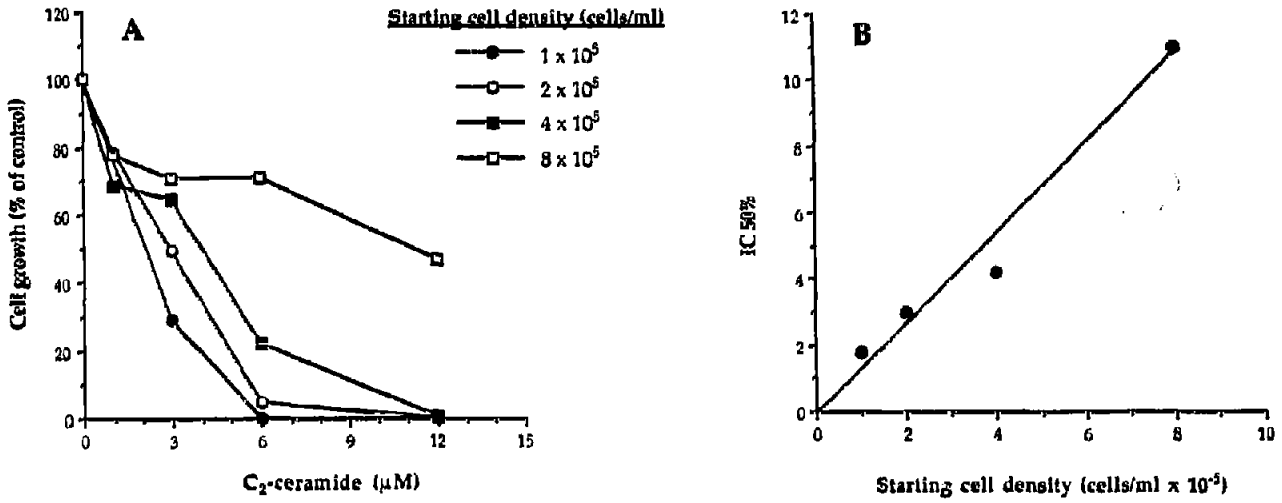


Fig. 2. Modulation of efficacy of $C_2$-ceramide by starting cell density. (A) HL-60 cells were suspended at different cell densities as indicated. At each starting cell density, HL-60 cells were treated with increasing concentrations of $C_2$-ceramide, and cell growth was monitored at 3 days. These results are representative of 3 experiments. (B) The concentration of $C_2$-ceramide producing 50% inhibition was plotted as a function of starting density of HL-60 cells. These results are representative of 3 experiments.

densities. In order to quantitate these effects, the concentration of ceramide required to produce 50% inhibition of cell growth was plotted as a function of starting cell density (Fig. 2B). These results show that ceramide potency is inversely proportional to starting cell density. Since the slope of the line is close to 1, a useful operational rule suggests that doubling cell density would require doubling ceramide concentrations to achieve the same effect. This rule, however, is limited by the solubility of ceramide in serum-free media. We have found that in the absence of serum proteins, the solubility of $C_2$-ceramide is approximately 10–15 $\mu$M (data not shown). Therefore, the experimental conditions to evaluate biologic activities of $C_2$-ceramide should be restricted to concentrations of $C_2$-ceramide below 10–15 $\mu$M. In turn, this defines the upper limit of starting cell densities, since progressively increasing cell density cannot be matched by increasing $C_2$-ceramide above 10–15 $\mu$M.

The progressive loss of ceramide potency with increasing starting cell density suggests that the potency of ceramide is determined primarily by the *cell concentration* of ceramide rather than the *bulk molar concentration*. This is expected for a molecule that presumably acts in membranes rather than in the fluid phase, as was shown with sphingosine effects on protein kinase C [4,8,9]. Therefore, when the concentrations of ceramide were recalculated as moles of $C_2$-ceramide per cell, the potency of ceramide appeared to be hardly modulated by starting cell density (data not shown). The $IC_{50\%}$ was approximately 10–20 fmol/cell at all cell densities evaluated. Therefore, in designing experiments aimed at discerning biologic activities of $C_2$-ceramide, the initial concentration range should be defined by the ratio of $C_2$-ceramide to starting cell density rather than by the bulk molar concentration.[1]

The potency of $C_2$-ceramide was dramatically modulated by the presence of fetal calf serum during treatment of HL-60 cells (Fig. 3). Thus, $C_2$-ceramide became much less potent and the useful concentration range at a given cell density was shifted approximately one log higher so that the $IC_{50\%}$ became approximately 30 $\mu$M. This is presumably due to the presence of serum proteins (especially albumin) that may act to bind $C_2$-ceramide, thus reducing its effective concentration and increasing its solubility. This was borne out when $C_2$-ceramide was delivered in a 1:1 complex with fatty acid-free bovine serum albumin to cells grown in the absence of fetal calf serum. Under these conditions, the dose-dependence on $C_2$-ceramide was also shifted significantly to the right and resembled that seen in the presence of fetal calf serum (Fig. 3).

## 4. DISCUSSION

The results from this study offer a better understanding of parameters that determine biologic activities of ceramide.

$C_2$-ceramide is an amphipathic 'cell-permeable' analog of ceramide which has become instrumental in defining biologic activities of ceramide [2,3]. The delineation of parameters that determine its pharmacologic/biologic application should therefore enhance the ability to determine further biologic activities of ceramide.

[1] Since this parameter most probably reflects the partitioning of ceramide into membranes, the effective concentration of $C_2$-ceramide (expressed as mol/cells) may also vary between different cell types depending on cell size, the total membrane surface per cell, and total lipid content of the cells [10].

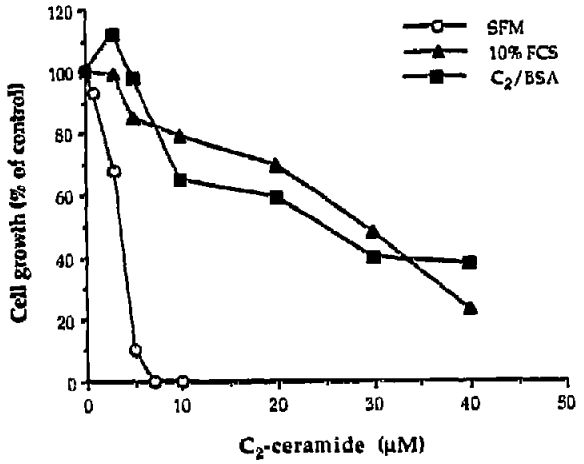


Fig. 3. Modulation of effects of $C_2$-ceramide by proteins. HL-60 cells were treated with the indicated concentrations of $C_2$-ceramide delivered to cells grown in serum-free media (SFM), or to cells grown in 10% fetal calf serum (10% FCS). $C_2$-ceramide was also delivered as a 1:1 complex with bovine serum albumin ($C_2$/BSA) to cells grown in serum-free media. Cell counts were determined at 3 days following treatment.

Moreover, these parameters should facilitate the examination of potential intracellular molecular targets for the action of ceramide.

The current studies have identified cell density and the presence of ceramide-binding proteins as very important determinants of the potency of ceramide. Thus, the effective concentration of ceramide is determined by the ratio of total (free) ceramide to total cell number, and the free effective concentration of ceramide is markedly influenced by the presence of ceramide-binding proteins such as bovine serum albumin or fetal calf serum.[2] These same considerations appear to apply in the determination of the action of sphingosine whereby the effectiveness of sphingosine is modulated by cell number and by the presence of serum proteins [4,5].

At this point, we may begin to generalize these considerations to the use of many amphiphilic compounds which include a number of pharmacologic reagents and an increasing number of endogenous cell regulatory molecules such as diacylglycerols and fatty acids. Attention to these factors is critical for the appropriate use of lipid mediators as regulators of cell function.

[2] A standard component of most cell culture growth media.

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