RESEARCH COMMUNICATION

Evidence against an early signalling role for ceramide in Fas-mediated apoptosis

Daniel J. SILLENCE* and David ALLAN

Department of Physiology, University College London, Rockefeller Building, University Street, London WC1E 6JJ, U.K.

We have investigated whether the increases in ceramide levels that occur during apoptosis in SKW 6.4 cells induced by anti-Fas antibody depend on the activation of caspases. Using cells prelabelled to equilibrium with [14 C]acetate, it was shown that the amount of ceramide approximately doubled after 24 h incubation with anti-Fas, but the time course of ceramide changes was slower than that of anti-Fas-induced cell death. Complete inhibition of the effects of anti-Fas on cell death and on ceramide production was observed when the caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-(*O*-methyl)fluoromethane (zVAD.fmk) was added together with anti-Fas, but *N*-

benzyloxycarbonyl-Phe-Ala-fluoromethane (a structurally similar cathepsin B inhibitor) had no effect. Treatment of cells with the Ca^{2+} -ionophore A23187 also doubled ceramide levels, but in this case the effect was complete within 2 h, was not blocked by zVAD.fmk and was not associated with increases in nuclear fragmentation. These results suggest that ceramide is not an upstream messenger in Fas-mediated apoptosis and may instead be produced as a consequence of processes downstream of the activation of caspases and increases in cytosolic calcium concentration.

INTRODUCTION

Programmed cell death or apoptosis, where cells activate an intrinsic death programme, has recently been recognized as a widespread and physiologically important phenomenon. One of the primary mediators of apoptosis in mammals is Fas, a receptor on the cell surface whose trimerization by Fas ligand [1] activates a series of aspartate-directed proteases (caspases) with similarity to the interleukin-1 β -converting enzyme (ICE) family which seem to play an integral part in the signalling processes leading to apoptosis [2]. Fas trimerization also activates a plasma-membrane or lysosomal sphingomyelinase, causing the generation of ceramide which may also be involved in control of the apoptotic process [3–20]. This interpretation has been supported by the observation that exogenous sphingomyelinase or short-chain ceramides promote apoptosis in various cell types, although this finding does not by itself confirm an essential role for ceramide in apoptosis. One thing which has been unclear is the temporal relationship between activation of caspases and activation of sphingomyelinase; certainly it is important to know which event comes first and how they are connected with the other changes which occur, such as membrane blebbing, rises in cytosolic Ca^{2+} levels, externalization of phosphatidylserine and nuclear degradation [21,22]. In the present work we sought to characterize Fas-mediated and Ca^{2+} -mediated ceramide changes in the absence or presence of the caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-(*O*-methyl)fluoromethane (zVAD.fmk), which is able to block apoptosis in SKW 6.4 and U937 cells [23]. The results suggest that ceramide production occurs as a downstream consequence of either activation of caspases after exposure to Fas-ligand or increases in cytosolic Ca^{2+} .

MATERIALS AND METHODS

Materials

Sodium [$2\text{-}^{14}\text{C}$]acetate was from Amersham International

(Amersham, Bucks., U.K.). Tissue-culture media and supplements were obtained from Gibco. Silica Kiesegel 60 TLC plates were obtained from Merck. Lipid standards and the Ca^{2+} ionophore A23187 were obtained from Sigma. Organic solvents were analytical grade and obtained from BDH. zVAD.fmk and *N*-benzyloxycarbonyl-Phe-Ala-fluoromethane (zFA.fmk) were from Enzyme Systems Products, Inc. (Dublin, CA, U.S.A.). Stock solutions of peptide inhibitors (25–100 mM) were prepared in dry DMSO and stored in aliquots at -80°C . Working dilutions were made in cell-culture medium immediately before use. Anti-Fas was kindly provided by Dr. Peter Krammer (DFDZ, Heidelberg, Germany).

Tissue culture

SKW 6.4 cells, a human B-cell lymphoma cell line, and U937 cells, a promyelocytic leukaemia cell line, were maintained in RPMI-1640 medium containing 25 mM Hepes and GlutaMAX 1 supplemented with penicillin/streptomycin and 10% heat-inactivated foetal-calf serum.

Cell labelling

SKW 6.4 cells were grown in the presence of 60 μCi of [^{14}C]acetate for 48 h and then portions (600 μl ; 1.5×10^4 cells) were incubated in the presence of 50 ng/ml anti-Fas or 5 $\mu\text{g}/\text{ml}$ A23187 for the times indicated in the Figure legends. Some samples of cells were treated additionally with 20 μM zVAD.fmk or zFA.fmk.

Lipid analysis

After removal of 100 μl of the cell suspension for the assay of apoptosis, incubations were terminated by the addition of 1.9 ml of chloroform/methanol (1:2, v/v) to the remaining cells. Aqueous and organic phases were then split according to the

Abbreviations used: zVAD.fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-(*O*-methyl)fluoromethane; zFA.fmk, *N*-benzyloxycarbonyl-Phe-Ala-fluoromethane; ICE, interleukin-1 β -converting enzyme; MAP kinase, mitogen-activated protein kinase.

* To whom correspondence should be sent.

procedure of Bligh and Dyer [24]. The chloroform phase was dried using a water pump, and neutral lipids were separated by TLC in the solvent system benzene/diethyl ether/ethanol/acetic acid (500:400:20:1, by vol.). Spots were imaged overnight on ^{14}C phosphorimager screens and quantified on a Fuji phosphor-image analyser. After this analysis of the neutral lipids the phospholipids were then separated by running the same plate in chloroform/methanol/acetic acid/water (75:45:12:2, by vol.) and repeating the phosphorimage analysis for the phospholipid spots. In some cases, lipid spots on TLC plates were located by reference to the phosphorimage and scraped into vials. After addition of 250 μl of chloroform/methanol/water (5:11:5, by vol.) and 5 ml of Hi-safe 3 scintillant (Pharmacia) the samples were counted for radioactivity in a Canberra Packard liquid-scintillation counter.

Apoptosis assay

Apoptosis in fixed cultured cells was assessed by measuring the proportion of cells possessing fragmented nuclei after staining with bisbenzimidazole (HOECHST 33258) as described previously [22]. A 100 μl portion of suspended cells was transferred to a Falcon 96-well plate. After centrifugation (700 g for 5 min) the cells were fixed in 50 % acetic acid/methanol (1:3, v/v) in PBS, followed by 100 % acetic acid/methanol (1:3, v/v) (with centrifugation at each step). The fixative was removed and the plates allowed to dry. Dried plates were stored at room temperature until they were stained by adding 100 μl of PBS containing 0.1 $\mu\text{g}/\text{ml}$ HOECHST 33258. Nuclei were assessed in an Olympus OMT2 inverted fluorescence microscope, using a long-working-distance objective at a final magnification of 400 $\times$.

RESULTS

Time course for ceramide changes during Fas-mediated apoptosis

The basal level of ceramide in SKW cells was 1.5 ± 0.1 % of total cholesterol levels, or 0.4 % of total phospholipid, which was about half of that reported previously in this cell type using the diacylglycerol kinase mass assay [3]. This value rose by 50 % after 8 h and by 100 % after 24 h following the addition of anti-Fas, whereas, in the absence of anti-Fas, ceramide rose by only 20 % in 24 h (Figure 1). The kinetics of ceramide production seemed to be slightly slower than those of cell death, which increased to 50 % at 4 h and close to 100 % at 24 h, and contrasted sharply with the rapidity of ceramide formation relative to cell death reported by other investigators [4,9,12,25]. We repeated this experiment with U937 cells, which have previously been reported to respond rapidly to anti-Fas with a large increase in the mass of ceramide measured with *Escherichia coli* diacylglycerol kinase [4]. However, like the authors of [26], we were unable to see any fast changes in ceramide (Figure 1) and saw rises in ceramide only at later times when cell death had increased markedly.

Time course for ceramide changes during treatment of SKW cells with Ca^{2+} /A23187

The above observation that the kinetics of the ceramide response to anti-FAS were slower than those of the induction of cell death suggested that such increases were due to an event downstream of the initial signalling and perhaps even a consequence of cell death itself. One downstream event following challenge of lymphocytes with anti-Fas is a sustained increase in cytosolic calcium [27,28] and, since it has previously been shown that sustained increases in cytosolic calcium using Ca^{2+} /A23187 lead

Figure 1 Ceramide increases and cell death as a consequence of treating SKW 6.4 cells with anti-Fas

SKW cells labelled with [^{14}C]acetate for 48 h were incubated with or without the addition of 50 ng/ml anti-Fas. At the indicated times, cell death and ceramide levels were determined as described in the Materials and methods section. The insert shows ceramide changes in SKW cells at shorter times. Ceramide data are expressed as a percentage of cholesterol radioactivity and are the means for four separate experiments. $\circ$, Ceramide (control); $\bullet$, ceramide (+ anti-Fas); $\square$, percentage nuclear fragmentation (control); $\blacksquare$, nuclear fragmentation (+ anti-Fas).

Figure 2 Time course of ceramide changes during Ca^{2+} /A23187 treatment of SKW 6.4 cells

[^{14}C]Acetate-labelled SKW cells were incubated in RPMI/Hepes with the addition of 5 $\mu\text{g}/\text{ml}$ A23187 for the indicated times. Ceramide and cell death (nuclear fragmentation) were quantified as described in the Materials and methods section. Results are the means ($\pm$ S.E.M.) for three experiments and are expressed as a percentage of cholesterol. $\circ$, Ceramide (control); $\bullet$, ceramide (+ A23187); $\square$, percentage nuclear fragmentation (control); $\blacksquare$, percentage nuclear fragmentation (+ A23187).

to activation of a sphingomyelinase in chicken erythrocytes [29,30], we wondered whether a similar effect would be observed in SKW cells. Figure 2 shows that treatment of SKW cells with calcium/A23187 over a 4 h period induced an increase in ceramide which was linear up to 2 h and reached a maximum similar to that seen during Fas-mediated apoptosis (about 100 %

Figure 3 Differential sensitivity of Fas- and Ca^{2+} /A23187-mediated ceramide increases to blockade by the caspase inhibitor zVAD.fmk

[^{14}C]Acetate-labelled SKW cells were challenged with 50 ng/ml anti-Fas in the presence or absence of 20 μM zVAD.fmk or zFA.fmk for 15 h. Where indicated SKW cells were incubated with 5 $\mu\text{g/ml}$ A23187 for 90 min. All incubations were done in the presence of 0.2% DMSO. Statistical significance: ** $P < 0.01$ compared with anti-Fas alone.

increase). However, in contrast with anti-Fas treatment, no increase in nuclear fragmentation was found to accompany the rise in ceramide (Figure 2).

Sensitivity of the Fas- and Ca^{2+} /A23187-mediated increases in ceramide to blockade by the caspase inhibitor zVAD.fmk

Experiments were carried out to determine whether the increases in ceramide that occur during Fas-mediated apoptosis depended on the activation of caspases. It was reasoned that a downstream ceramide response would be blocked by the caspase inhibitor zVAD.fmk, whereas one that occurred independently from caspase activation would be insensitive to this inhibitor. Figure 3 shows that inhibition of apoptosis by the addition of zVAD.fmk also blocked Fas-induced increases in ceramide, whereas the structurally related peptide zFA.fmk had no effect on either apoptosis, caspases or ceramide levels (Figure 3). In contrast, ceramide increases induced by treatment of cells with Ca^{2+} /A23187 were completely insensitive to blockade with the caspase inhibitor zVAD.fmk (Figure 3).

DISCUSSION

The results of this work show that ceramide levels double during Fas-mediated apoptosis and (more rapidly) during elevation of intracellular Ca^{2+} by A23187 treatment. Ceramide increases of a similar magnitude have been seen previously in cells undergoing apoptosis [3,12,26,33]. Considering that the resting level of ceramide is only about 0.4% of the total lipid, the rise is still only small, and if the extra ceramide is derived from sphingomyelin, then its production would require the breakdown of only about 10% of total cellular sphingomyelin. With anti-Fas or A23187, this appeared to be the maximum value, suggesting that, in both cases, only a small proportion of the total sphingomyelin was susceptible to breakdown.

Scheme 1 Possible sequence of events leading to ceramide generation during Fas-mediated apoptosis

In none of these experiments with anti-Fas was a measurable increase in ceramide seen at early times (2–20 min), so that ceramide production was unlikely to be closely linked to receptor activation in this case, in contrast with many other instances reported in the literature [4,9,12,25,31]. Indeed, ceramide increased with kinetics slower than the increases in nuclear fragmentation (Figure 1).

The observation that Fas-mediated increases in ceramide were blocked by the caspase inhibitor zVAD.fmk complements the work of Garmen et al. [26], who showed that the ceramide response to anti-Fas was blocked by a tetrapeptide inhibitor of CPP32-like protease (caspase-3) [35]. These data, together with ours, therefore place the generation of ceramide downstream of the activation of two types of signalling protease. Although calcium influx stimulated ceramide accumulation to a similar extent to anti-Fas, the effect was much more rapid (2 h compared with 24 h) and was not inhibited by zVAD.fmk. This result suggests that Ca^{2+} -induced increases in ceramide levels were not dependent on the activation of caspases. Since nuclear fragmentation was not increased by A23187 treatment (Figure 2), it appears unlikely that ceramide accumulation necessarily induces the onset of apoptosis in all cell types, as has been suggested previously [3–20,30,32]. Indeed we have found that baby hamster kidney cells are able to grow even in the presence of sphingomyelinase, a treatment which induces large sustained increases in plasma-membrane ceramide (D. J. Silence and D. Allan, unpublished work). Increases in cytoplasmic Ca^{2+} do occur during Fas-mediated apoptosis [21,27,28,33] and the Ca^{2+} ionophore A23187 can induce nuclear fragmentation in some cell types [21,34], but a 4 h treatment with A23187 does not induce nuclear fragmentation in SKW cells, even when the ceramide increase is maximal (Figure 2). Hence our observations suggest that increases in Ca^{2+} and ceramide are downstream events in apoptosis and that, probably, production of ceramide depends on the increase in Ca^{2+} . Overall, the results are consistent with a model (Scheme 1) whereby the activation of caspases stimulates a rise in intracellular Ca^{2+} which then leads to the activation of

a sphingomyelinase perhaps similar to that in chicken erythrocytes [29,30].

Whether the ceramide produced during apoptosis has subsequent signalling functions that complement the activation of caspases and the rise in Ca^{2+} levels has not been addressed in the present study. Many possible targets for ceramide signalling have been found, such as the activation of NF- κ B [31] and the activation of the mitogen-activated protein (MAP) kinase cascade via a ceramide-activated protein kinase ('CAPK') or phosphatase ('CAPP') [32,33]. It should be mentioned, however, that alternative pathways for the activation of MAP kinase and NF- κ B by both tumour-necrosis factor and Fas have also been proposed via 'death' adaptor molecules and NF- κ B-inducing kinase ('NIK') [36]. In any case, the slow (and probably Ca^{2+} -dependent) increases in ceramide reported here during anti-Fas treatment of SKW cells seem less likely to have a role in the early events of apoptosis.

We thank Dr. Mike Jacobson and Professor Martin Raff for advice and supplying zVAD.fmk. We also thank Dr. Peter Krammer for supplying anti-Fas. This work was supported by the Medical Research Council.

REFERENCES

- Nagata, S. and Golstein, P. (1995) *Science* **267**, 1449–1456
- Fraser, A. and Evan, G. (1996) *Cell* **85**, 781–784
- Tepper, C. G., Jayadev, S., Liu, B., Bielawska, A., Wolff, R., Yonehara, S., Hannun, Y. A. and Seldin, M. F. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8443–8447
- Cifone, M. G., De, M. R., Roncalioli, P., Rippo, M. R., Azuma, M., Lanier, L. L., Santoni, A. and Testi, R. (1994) *J. Exp. Med.* **180**, 1547–1552
- Linardic, C. M. and Hannun, Y. A. (1994) *J. Biol. Chem.* **269**, 23530–23537
- Zhang, J., Alter, N., Reed, J. C., Borner, C., Obeid, L. M. and Hannun, Y. A. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5325–5328
- Wright, S. C., Zheng, H. and Zhong, J. (1996) *FASEB J.* **10**, 325–332
- Wiesner, D. A. and Dawson, G. (1996) *J. Neurochem.* **66**, 1418–1425
- Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Szabo, E., Zon, L. I., Kyriakis, J. M., Haimovitz, F. A., Fuks, Z. and Kolesnick, R. N. (1996) *Nature (London)* **380**, 75–79
- Venable, M. E., Lee, J. Y., Smyth, M. J., Bielawska, A. and Obeid, L. M. (1995) *J. Biol. Chem.* **270**, 30701–30708
- Saba, J. D., Obeid, L. M. and Hannun, Y. A. (1996) *Philos. Trans. R. Soc. London B Biol. Sci.* **351**, 233–240
- Pronk, G. J., Ramer, K., Amiri, P. and Williams, L. T. (1996) *Science* **271**, 808–810
- Martin, S. J., Newmeyer, D. D., Mathias, S., Farschon, D. M., Wang, H. G., Reed, J. C., Kolesnick, R. N. and Green, D. R. (1995) *EMBO J.* **14**, 5191–5200
- Karasavvas, N., Erukulla, R. K., Bittman, R., Lockshin, R. and Zakeri, Z. (1996) *Eur. J. Biochem.* **236**, 729–737
- Brugg, B., Michel, P. P., Agid, Y. and Ruberg, M. (1996) *J. Neurochem.* **66**, 733–739
- Chang, Y., Abe, A. and Shayman, J. A. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 12275–12279
- Latini, K. M. and Koretzky, G. A. (1996) *Blood* **87**, 871–875
- Nickels, J. T. and Broach, J. R. (1996) *Genes Dev.* **10**, 382–394
- Sawai, H., Okazaki, T., Yamamoto, H., Okano, H., Takeda, Y., Tashima, M., Sawada, H., Okuma, M., Ishikura, H., Umehara, H. et al. (1995) *J. Biol. Chem.* **270**, 27326–27331
- Chinnaiyan, A. M., Tepper, C. G., Seldin, M. F., O'Rourke, K., Kischkel, M. C., Hellbardt, H., Krammer, P. H., Peter, M. E. and Dixit, V. M. (1996) *J. Biol. Chem.* **271**, 4961–4965
- Vanags, D. M., Pörn-Ares, M. I., Coppola, S., Burgess, D. H. and Orrenius, S. (1996) *J. Biol. Chem.* **271**, 31075–31085
- McConkey, D. J. and Orrenius, S. (1996) *J. Leukocyte Biol.* **59**, 775–783
- Jacobson, M. D., Weil, M. and Raff, M. C. (1996) *J. Cell Biol.* **133**, 1041–1051
- Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Obeid, L. M., Linardic, C. M., Karolak, L. A. and Hannun, Y. A. (1993) *Science* **259**, 1769–1771
- Garmen, S., Marzo, I., Anel, A., Pineiro, A. and Naval, J. (1996) *FEBS Lett.* **390**, 233–237
- Oshimi, Y. and Miyazaki, S. (1995) *J. Immunol.* **154**, 599–609
- Rovere, P., Clementi, E., Ferrarini, M., Helti, S., Sciorati, C., Sabbadini, M. G., Rugarli, C. and Manfredi, A. A. (1996) *J. Immunol.* **156**, 4631–4637
- Allan, D. and Raval, P. J. (1987) *Biochim. Biophys. Acta* **897**, 355–363
- Allan, D., Thomas, P. and Limbrick, A. R. (1982) *Biochim. Biophys. Acta* **693**, 53–67
- Wiegmann, K., Schutze, S., Machleidt, T., Witte, D. and Kronke, M. (1994) *Cell* **78**, 1005–1015
- Adam-Klages, S., Adam, D., Wiegmann, K., Struve, S., Kolanus, W., Schneider-Mergener, J. and Kronke, M. (1996) *Cell* **86**, 937–947
- Hannun, Y. A. (1996) *Science* **274**, 1855–1859
- Zhivotovsky, B., Nicotera, P., Bellomo, G., Hanson, K. and Orrenius, S. (1993) *Exp. Cell. Res.* **207**, 163–170
- Enari, M., Talianian, R., Wong, W. and Nagata, S. (1996) *Nature (London)* **380**, 723–726
- Malinin, N. L., Boldin, M. P., Kovalenko, A. V. and Wallach, D. (1997) *Nature (London)* **385**, 540–544