Chapitre I – L'accumulation de céramides C_{16} et C_{24} par la voie *de novo* contribue à l'apoptose spontanée des neutrophiles

Une fois libérés dans la circulation, les neutrophiles entament spontanément un processus de mort par apoptose. En effet, après avoir passé quelques heures dans la circulation, apparaissent à leur surface les stigmates morphologiques de l'apoptose précoce tels que l'externalisation de la PS. Les neutrophiles vieillissants sont ainsi reconnus et phagocytés par les macrophages spléniques. Ce phénomène permet une élimination "sans traces" des neutrophiles et évite toute libération spontanée du contenu cytotoxique, et par conséquent pro-inflammatoire, dans la circulation. A l'heure actuelle, bien que plusieurs hypothèses aient été formulées, personne n'a encore pu démontrer précisément les mécanismes moléculaires impliqués dans l'apoptose spontanée des neutrophiles. Les céramides sont des sphingolipides reconnues pour leur rôle de messagers secondaires dans de nombreux processus biologiques tels que la différentiation cellulaire, la sénescence et l'apoptose. En particulier, dans les neutrophiles, il a été montré que les céramides facilitaient l'activation de la caspase-8 suite à la stimulation des cellules par FasL et à la formation du complexe multi-protéique DISC (Scheel-Toellner *et al*., 2004a ; 2004b). Dans cette étude nous démontrons le rôle important joué par les céramides de type C_{16} et C_{24} dans l'apoptose spontanée des neutrophiles fraîchement isolés de la circulation sanguine et placés en culture.

Le taux intracellulaire des différents types de céramides a été mesuré par spectrométrie de masse, grâce à une technique développée à l'Université de Liège par Fillet et collaborateurs (2002). Le taux d'apoptose a été mesuré par la technique usuelle du double marquage à l'annexine-V-FITC et à l'iodure de propidium ; les cellules ont été analysées par cytométrie de flux (FACSAria). Afin d'éviter une lyse cellulaire lors de l'analyse, nous avons opté pour une technique *in situ* pour mesurer l'activité des caspases-8, -9, -3. A un temps donné, les neutrophiles ont été traités avec des peptides substrats, inhibiteurs irréversibles et spécifiques des différents types de caspases, couplés à des molécules fluorogènes. Apres une heure d'incubation avec ces peptides, les cellules ayant conservé leur intégrité étaient analysées par cytométrie de flux. Le suivi au cours du temps des taux intracellulaires des différents types de céramides a montré que les céramides de type C_{16} et C_{24} s'accumulaient spontanément dans les neutrophiles placés en culture. Cette accumulation précédait l'externalisation de la PS ainsi que l'activation des caspases-8, -9 et -3.

En traitant les granulocytes avec des inhibiteurs spécifiques des différentes voies de biosynthèse des céramides, nous avons montré que les céramides C_{16} et C_{24} étaient générées dans les neutrophiles par la voie *de novo* à partir de la condensation de l'acide aminé sérine et d'un groupement acyle, dérivé d'un acide gras, couplé au coenzyme A. Par contre, nous avons montré que l'autre voie majeure de biosynthèse des céramides, appelée voie de recyclage de la SM, n'était pas impliquée dans l'accumulation des céramides C_{16} et C_{24}. Toujours grâce à l'utilisation de ces molécules pharmacologiques, nous avons montré que l'augmentation ou la diminution des taux endogènes de C_{16} et C_{24} augmentait et diminuait, respectivement, les taux d'apoptose et d'activation des caspases-8, -9 et -3 dans les neutrophiles. Ces résultats ont été confirmés en ajoutant, au milieu de culture, des céramides C_{16} et/ou C_{24} exogènes. En effet, ce traitement augmentait significativement le taux d'apoptose des neutrophiles. Enfin, nous avons traité des neutrophiles avec du GM-CSF et avons montré que cette cytokine anti-apoptotique bloquait l'accumulation des céramides C_{16} et C_{24} dans les

neutrophiles. Au contraire, lorsque des neutrophiles étaient traités avec FasL, une molécule pro-apoptotique, les taux endogènes des céramides C_{16} et C_{24} n'étaient pas modifiés.

***De novo* C16- and C24-ceramide generation contributes to spontaneous neutrophil apoptosis**

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De novo C_{16}- and C_{24}-ceramide generation contributes to spontaneous neutrophil apoptosis

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Abstract: Neutrophils rapidly undergo spontaneous apoptosis following their release from the bone marrow. Although central to leukocyte homeostasis, the mechanisms that regulate neutrophil apoptosis remain poorly understood. We show here that apoptosis of cultured neutrophils is preceded by a substantial increase in the intracellular levels of 16 and 24 carbon atom (C_{16}- and C_{24})-ceramides, which are lipid second messengers of apoptosis and stress signaling. Treatment of neutrophils with fumonisin B_2, a selective inhibitor of the de novo pathway of ceramide synthesis, prevented accumulation of C_{16}- and C_{24}-ceramides. Moreover, fumonisin B_2 significantly reduced caspase-3, -8, and -9 activation and apoptosis in these cells. Conversely, 3-O-methylsphingomyelin and fantofarone, which are specific inhibitors of neutral and acid sphingomyelinases, respectively, neither inhibited C_{16}- and C_{24}-ceramide production nor decreased the apoptosis rate in neutrophils, indicating that in these cells, ceramides are not generated from membrane sphingomyelin. Further experiments showed that increasing endogenous C_{16}- and C_{24}-ceramide levels by using DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol and (1S,2R)-D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol, two inhibitors of ceramide metabolism, enhances caspase-3, -8, and -9 activity and increases neutrophil apoptosis. Similarly, apoptosis was induced rapidly when synthetic C_{16}- and/or C_{24}-ceramides were added to neutrophil cultures. Finally, GM-CSF, a cytokine that delays neutrophil apoptosis, abrogated C_{16}- and C_{24}-ceramide accumulation totally in cultured neutrophils, whereas Fas ligation accelerated apoptosis in these cells without affecting de novo ceramide production. We conclude that de novo generation of C_{16}- and C_{24}-ceramides contributes to spontaneous neutrophil apoptosis via caspase activation and that GM-CSF exerts its antiapoptotic effects on neutrophils, at least partly through inhibition of ceramide accumulation. *J. Leukoc. Biol.* 81: 000–000; 2007.

Key Words: *caspases · GM-CSF · granulocytes · survival*

INTRODUCTION

Neutrophils represent 60% of the total circulating leukocytes in humans and play a central role in host defense against infectious microorganisms. Of all the cells of the immune system, the neutrophil has the shortest half-life, estimated to be between 6 h and 18 h [1, 2]. After release into the bloodstream, mature neutrophils rapidly undergo spontaneous apoptosis and are eliminated through phagocytosis by spleen, bone marrow, and liver macrophages [1–3]. The consequence of the short half-life of circulating neutrophils is that 10^{11} neutrophils need to be produced daily in the bone marrow to maintain homeostasis [4]. The reasons why bloodstream neutrophils die rapidly are not clear but may be related to the capacity of these cells to inflict tissue damage if they are activated inappropriately [1]. In response to pathogen invasion, neutrophils migrate into peripheral tissues, where they kill pathogens using several microbicidal mechanisms, including phagocytosis, production of reactive oxygen intermediates, and release of proteases [5, 6]. To optimize pathogen elimination, the lifespan of extravasated neutrophils is extended by a range of inflammatory mediators, including cytokines such as GM-CSF [7] and bacterial components including LPS [8]. After killing the ingested microorganisms, the neutrophils enter apoptosis and are phagocytosed by resident macrophages, preventing loss of neutrophil contents and tissue destruction [3, 9]. It thus appears that regulation of neutrophil lifespan is essential to prevent tissue damage and to ensure the maintenance of neutrophil homeostasis, the efficient removal of pathogens, and the resolution of the inflammatory process. However, the molecular mechanisms involved in the control of neutrophil apoptosis remain poorly understood.

Ceramides are bioactive sphingolipids, which mediate antiproliferative responses such as cell cycle arrest, senescence, and apoptosis [10, 11]. The most common ceramides have acyl chain lengths of 16–24 carbon atoms (C_{16}–C_{24}) and are produced in response to various stress stimuli, including TNF-α, Fas ligand (FasL), oxidative stress, anticancer drugs, ionizing

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radiation, and ultraviolet light [10, 11]. Ceramides are generated from the major membrane sphingolipid sphingomyelin by acid and neutral sphingomyelinases (ASMase and NSMase) or through the de novo synthesis pathway [11–13]. De novo ceramide biosynthesis requires coordinate action of serine palmitoyl transferase and ceramide synthase to generate ceramides. Serine palmitoyl transferase catalyzes the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine, which is reduced to sphinganine and subsequently, N-acylated by ceramide synthase to form dihydroceramide. Finally, a double bond is introduced by dihydroceramide desaturase to produce ceramides. However they are formed, accumulating ceramides may induce cytochrome c release, caspase activation, and apoptosis [10, 11]. Once generated, ceramides may be converted into a variety of metabolites [12, 13]. Phosphorylation by ceramide kinase generates ceramide 1-phosphate, and deacylation by ceramidases yields sphingosine. Ceramides may also be converted back to sphingomyelin by sphingomyelin synthase. Finally, ceramides can be glycosylated by glucosylceramide synthase to form glucosylceramides.

It has been shown previously that the apoptotic program may be initiated in neutrophils through reactive oxygen species (ROS)-induced ASMase activation and subsequent ceramide accumulation [14]. In the present study, we have further investigated the role of ceramides in regulating neutrophil apoptosis and show that de novo synthesis of C_{16}- and C_{24}-ceramides is a crucial event in the induction of spontaneous neutrophil death. Moreover, we demonstrate that GM-CSF reduces accumulation of de novo-generated C_{16}- and C_{24}-ceramides in neutrophils, a mechanism that could account for the antiapoptotic function of this cytokine.

MATERIALS AND METHODS

Cell sorting, culture, and treatment

Human blood neutrophils, lymphocytes, and monocytes were obtained from buffy coats (Transfusion Center, Liege, Belgium). Neutrophils were separated from mononuclear cells by density centrifugation (Histopaque, Sigma-Aldrich, Bornem, Belgium). Contaminating erythrocytes were removed from the neutrophil fraction by hypotonic lysis. Lymphocytes and monocytes were isolated from the mononuclear fraction by positive magnetic selection using microbeads coated with anti-CD3 and anti-CD22 antibodies (lymphocytes) or anti-CD14 antibodies (monocytes) (CD3, CD22, and CD14 MicroBeads, Miltenyi Biotec, Paris, France). Neutrophil, lymphocyte, and monocyte purity, as determined by counting of cytospin preparations stained with Diff-Quick (Dade Behring, Dudingen, Germany), was always >95%. Blood cells were cultured at a density of 2×10^6 cells/ml in RPMI 1640 supplemented with 1% glutamine, 10% FCS, 50 μg/ml streptomycin, and 50 IU/ml penicillin (all from Gibco-BRL, Grand Island, NY, USA). HCT-116, human embryonic kidney (HEK)-293, HeLa, ovarian carcinoma (OVCAR), Phoenix, Jurkat, and Raji cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). These cell lines were cultured in DMEM (HeLa, HEK-293, and Phoenix cells), McCoy's 5A (HCT-116 cells), or RPMI 1640 (OVCAR, Jurkat, and Raji cells), supplemented with 1% glutamine, 10% FCS, 50 μg/mL streptomycin, and 50 IU/mL penicillin. Neutrophils were cultured in the presence or absence of human recombinant GM-CSF (PeproTech EC, London, UK), fumonisin B_2 (Biomol, Plymouth Meeting, PA, USA), 3-O-methylsphingomyelin (3-OMS; Biomol), fantofarone (SR33557; Sanofi Pharm. N.V., Brussels, Belgium), DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol.HCl (PPMP; Biomol), (1S,2R)-D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol (MAPP; Biomol), synthetic C_{16}- or C_{24}-ceramides (Acros Organics, Geel, Belgium), or soluble FasL (sFasL; PeproTech EC). GM-CSF and sFasL were dissolved in PBS. Fumonisin B_2 and PPMP were dissolved in water. MAPP, 3-OMS, and fantofarone were dissolved in ethanol. C_{16}- and C_{24}-ceramides were dissolved in ethanol/dodecan (99.8/0.2, v/v). An equal amount of solvent was always added to control cultures.

Measurement of intracellular ceramide levels

Liquid chromatography-electrospray ionization tandem-mass spectrometry (LC-ESI-MS/MS) was used to measure intracellular ceramide levels as described previously [15]. Standard solutions for optimization of LC-ESI-MS/MS were prepared by dissolving ceramides in a 99.8/0.2 (v/v) mixture of ethanol/formic acid to reach a concentration of 1 μg/ml. For extraction of cellular lipids, cells were rinsed twice with ice-cold PBS, then scraped in PBS, and centrifuged at 800 *g*. The resulting pellet was homogenized in distilled water by sonication. An aliquot of the cell homogenate was reserved for determination of protein levels. C_{12}-ceramide (10 ng; as an internal standard) was added to samples of cell lysates containing 300 μg protein. Lipids were extracted using Folch's partition with a mixture of chloroform and methanol (2/1, v/v). Samples were then centrifuged at 1500 *g* and washed with chloroform, methanol, and water (5/48/47, v/v/v). The organic phase was evaporated to near dryness under a gentle stream of dry nitrogen. The samples were reconstituted by vortexing with 100 μl of a mixture of ethanol/formic acid (99.8/0.2) until they were dissolved completely. To avoid any loss of lipids, the entire procedure was performed in siliconized glassware. The LC-ESI-MS/MS procedure we used detects endogenous ceramides C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, and C_{24} [15]. The levels of each ceramide species were expressed as their relative abundance compared with C_{16}-ceramide level in untreated neutrophil.

Detection of apoptosis by cytochemical staining

Apoptosis was assessed by staining with Annexin-V-FITC and propidium iodide (PI) using the Annexin-V-Fluos staining kit (Roche, Mannheim, Germany), following the recommendations of the manufacturer. Flow cytometry analyses were performed with a FACSAria™ (BD Biosciences, San Jose, CA, USA).

Assay of caspase activity

Caspase activities were determined using the CaspaTag™ caspase-3, caspase-8, and caspase-9 in situ assay kits (Chemicon International, Billerica, MA, USA). These are fluorochrome-labeled caspase inhibitors [sulforhodamine-labeled Asp-Glu-Val-Asp-fluoromethylketone (fmk) for caspase-3, fluorescein-labeled Leu-Glu-Thr-Asp-fmk for caspase-8, and fluorescein-labeled Leu-Glu-His-Asp-fmk for caspase-9), which are cell-permeable and nontoxic and covalently bind only active caspases. Analysis of caspase activity was performed using flow cytometry. Briefly, freshly isolated neutrophils and neutrophils that were cultured for 5 h in the absence or presence of 50 μM fumonisin B_2, 50 μM MAPP, or 30 μM PPMP were washed and resuspended in complete RPMI supplemented with fluorochrome-peptide-fmk for 1 h at 37°C under 5% CO_2. Cells were washed twice and resuspended in fixing buffer. The cells were analyzed immediately by flow cytometry.

Statistical analysis

Data are presented as means ± SD. The difference between mean values was estimated using an ANOVA with subsequent Fisher's protected least significant difference tests or a Student's *t*-test for unpaired data. A value of $P < 0.05$ was considered significant. All presented results are representative of at least three similar experiments.

RESULTS

Parallelism between C_{16}- and C_{24}-ceramide accumulation and triggering of apoptosis in cultured neutrophils

To determine the role of ceramides in spontaneous neutrophil apoptosis, human blood neutrophils were isolated from buffy coats and cultured for 0, 3, 6, 12, 18, and 24 h before

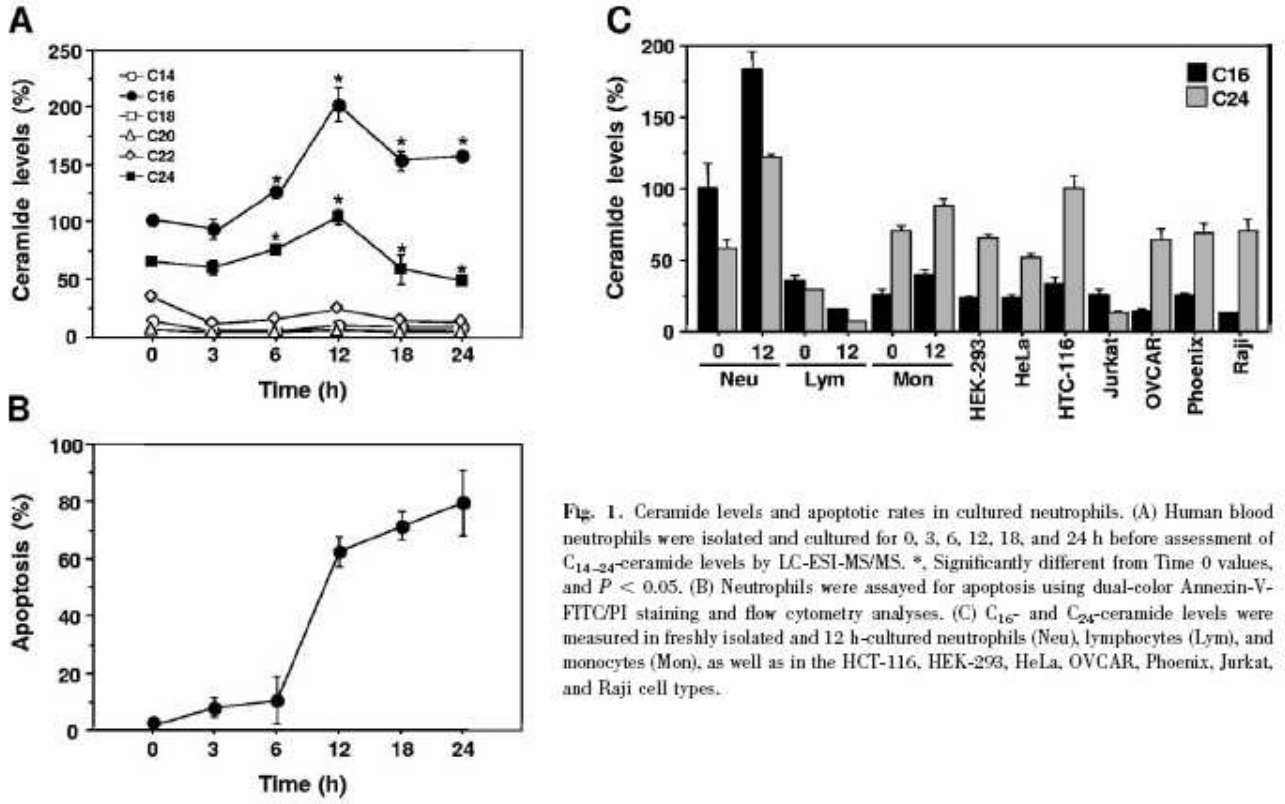

Fig. 1. Ceramide levels and apoptotic rates in cultured neutrophils. (A) Human blood neutrophils were isolated and cultured for 0, 3, 6, 12, 18, and 24 h before assessment of C_{14-24}-ceramide levels by LC-ESI-MS/MS. *, Significantly different from Time 0 values, and $P < 0.05$. (B) Neutrophils were assayed for apoptosis using dual-color Annexin-V-FITC/PI staining and flow cytometry analyses. (C) C_{16}- and C_{24}-ceramide levels were measured in freshly isolated and 12 h-cultured neutrophils (Neu), lymphocytes (Lym), and monocytes (Mon), as well as in the HCT-116, HEK-293, HeLa, OVCAR, Phoenix, Jurkat, and Raji cell types.

measurement of C_{14-24}-ceramide content by LC-ESI-MS/MS and assessment of apoptosis by the Annexin-V-FITC/PI detection method (**Fig. 1, A and B**). Figure 1A shows that high levels of C_{16}- and C_{24}-ceramides were detected in freshly isolated neutrophils. C_{16}- and C_{24}-ceramide rates remained unchanged until 3 h but then increased gradually to peak at 12 h. C_{24}-ceramide levels returned to baseline at 18 h, whereas C_{16}-ceramide rates decreased slightly to reach intermediate values at 18 h and 24 h. C_{14}-, C_{18}-, C_{20}-, and C_{22}-ceramides were barely detectable at any time-point. Freshly isolated neutrophils contained very few apoptotic cells (Fig. 1B). Apoptosis rates remained low until 6 h but increased dramatically at 12 h. At this time-point, ~62% of neutrophils were dead. The apoptotic rate then continued to increase gradually, reaching values of ~80% at 24 h. It is noteworthy that the increase in C_{16}- and C_{24}-ceramide levels (between 3 h and 12 h) just preceded the increase in neutrophil apoptosis (12 h; compare Fig. 1, A and B). The experiments shown in Figure 1, A and B, have been reiterated with neutrophils isolated from freshly sampled blood ("fresh neutrophils") rather than with "buffy coat neutrophils." Fresh neutrophils provided similar results than did buffy coat neutrophils (data not shown). Finally, the relative abundance of C_{16}- and C_{24}-ceramides in blood neutrophils were compared with those in various other cell types, namely, primary lymphocytes and monocytes, and the HCT-116, HEK-293, HeLa, OVCAR, Phoenix, Jurkat, and Raji cell lines. C_{24}-ceramide levels in neutrophils were comparable with those in the other cell types, except that only low C_{24}-ceramide rates were found in freshly isolated and 12 h-cultured, primary lymphocytes (Fig. 1C). Conversely, C_{16}-ceramide levels were much higher in neutrophils than in any of the other cell types examined (Fig. 1C). These results show that C_{16}-ceramide production is elevated in neutrophils and that C_{16}- and C_{24}-ceramide accumulation precedes apoptosis in these cells.

C_{16}- and C_{24}-ceramides are generated via the de novo pathway in neutrophils and contribute to spontaneous apoptosis of these cells

Ceramides can be generated from sphingomyelin through activation of SMases or from activation of the de novo pathway. To determine the source of C_{16}- and C_{24}-ceramides in neutrophils, we have treated cultured neutrophils with 3-OMS, fantofarone (SR33557), or fumonisin B_2. 3-OMS is a specific NSMase inhibitor [16], whereas fantofarone selectively blocks ASMase [17]. Fumonisin B_2 is a specific inhibitor of ceramide synthase, blocking the conversion of sphinganine to dihydroceramide in the de novo pathway [18, 19]. As depicted in **Figure 2A**, treatment of cultured neutrophils with fumonisin B_2 totally prevented C_{16}- and C_{24}-ceramide accumulation at 6 h and 12 h. C_{16}-ceramide levels even declined below the baseline in fumonisin B_2-treated neutrophils. Conversely, 3-OMS and fantofarone had no effect on ceramide production (Fig. 2A). Consistent with these observations, fumonisin B_2, but neither 3-OMS nor fantofarone, reduced the rate of neutrophil apoptosis significantly at 12, 18, and 24 h (Fig. 2B). Taken together, these results indicate that C_{16}- and C_{24}-ceramides generated via the de novo pathway accumulate in neutrophils, thereby contributing to the induction of spontaneous apoptosis. Moreover, it also appears that basal production of C_{16}-ceramide is at least partly dependent on the de novo pathway.

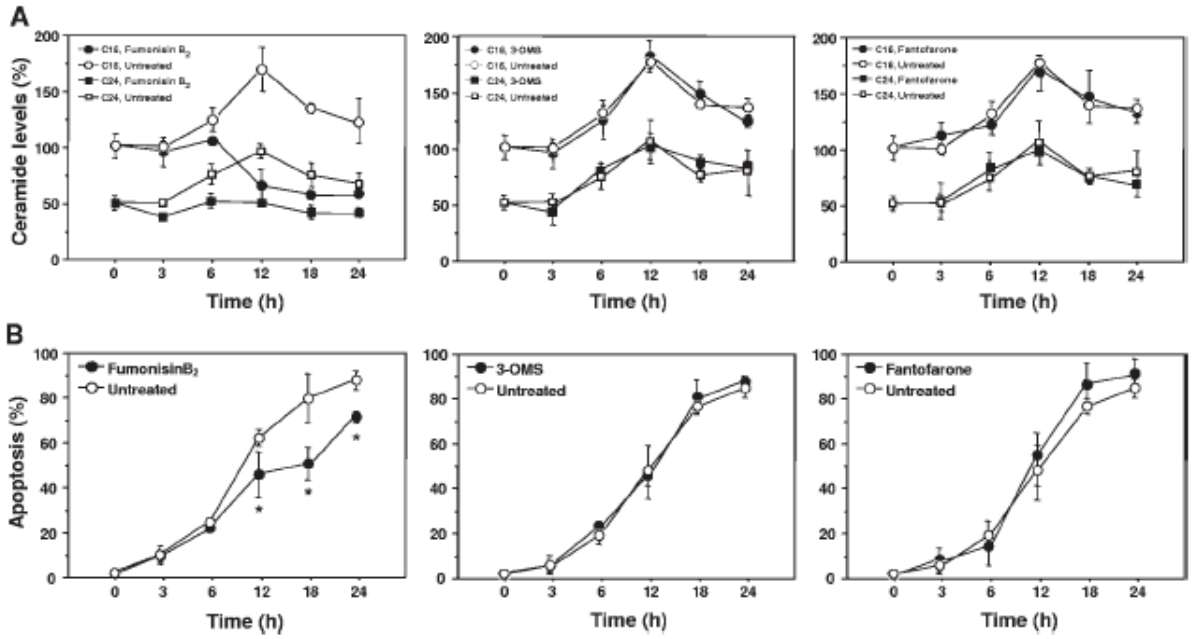

Fig. 2. Effects of fumonisin B_2, 3-OMS, and fantofarone (SR33557) on C_{16}- and C_{24}-ceramide levels and apoptotic rates. (A) Freshly isolated blood neutrophils were treated with 50 μM fumonisin B_2, 10 μM 3-OMS, or 10 μM fantofarone. Zero, 3, 6, 12, 18, and 24 h later, intracellular C_{16}- and C_{24}-ceramide levels were estimated by LC–ESI-MS/MS. Untreated cells were used as controls. (B) Apoptotic rates were measured at the different time-points by Annexin-V-FITC/PI staining and flow cytometry analyses. *, Significantly different from the values obtained with untreated cells ($P<0.05$).

Increasing endogenous C_{16}- and C_{24}-ceramide levels using inhibitors of ceramide metabolism increases neutrophil apoptosis

To further explore the role of ceramides in neutrophil apoptosis, we have amplified the endogenous ceramide levels in cultured neutrophils by using PPMP and MAPP, two inhibitors of ceramide metabolism. PPMP specifically inhibits glucosylceramide synthase, thus blocking ceramide glycosylation [20]. MAPP is a selective inhibitor of the alkaline ceramidase, which breaks down ceramides to sphingosine [21]. As shown in **Figure 3A**, PPMP increased C_{16}- and C_{24}-ceramide levels substantially in cultured neutrophils, whereas MAPP enhanced C_{16}-ceramide levels without affecting C_{24}-ceramide rates. The effects of PPMP and MAPP were observed as early as 3 h after treatment and were maintained until 24 h (Fig. 3A). For example, at 3 h, C_{16}- and C_{24}-ceramide levels in PPMP-treated cells were equal to or even greater than those measured in control cells at 12 h. Similarly, C_{16}-ceramide levels were higher in MAPP-treated cells at 3 h than in untreated cells at 12 h. Elevation of endogenous ceramide levels was paralleled by a significant increase in the apoptosis rates in PPMP- and MAPP-treated neutrophils, as compared with untreated cells (Fig. 3B), confirming that C_{16}- and C_{24}-ceramides may play an active role in inducing the apoptotic program in these cells.

Rapid onset of apoptosis in neutrophils treated with synthetic C_{16}- and C_{24}-ceramides

To ascertain that C_{16}- or C_{24}-ceramide accumulation is sufficient to induce neutrophil apoptosis, freshly isolated neutrophils were treated for 6 h with synthetic C_{16}- or C_{24}-ceramides before apoptosis was assessed by Annexin-V-FITC/PI staining. Treatment with synthetic C_{16}-ceramide significantly increased the intracellular levels of C_{16}-ceramide without changing the C_{24}-ceramide contents (**Fig. 4A**, left panel). Similarly, treatment with synthetic C_{24}-ceramide drastically enhanced the intracellular levels of C_{24}-ceramide (Fig. 4A, middle panel). The apoptotic rate was increased significantly in the presence of synthetic C_{16}- or C_{24}-ceramides (Fig. 4B). However, synthetic C_{16}-ceramide was more potent than C_{24}-ceramide in inducing neutrophil apoptosis (Fig. 4B, compare left and middle panels). Alternatively, neutrophils were treated with a combination of synthetic C_{16}- and C_{24}-ceramides. Addition of C_{16}- and C_{24}-ceramides led to a significant increase in intracellular levels of both ceramide species (Fig. 4A, right panel). Moreover, exogenously added C_{16}- and C_{24}-ceramides had an additive effect on neutrophil apoptosis (Fig. 4B, compare right panel with left and middle panels). These data establish that accumulation of C_{16}- and/or C_{24}-ceramides is sufficient to trigger neutrophil apoptosis.

De novo-generated C_{16}- and C_{24}-ceramides work upstream of caspase-3, -8, and -9 in neutrophils

Several studies have demonstrated a critical role for caspase-3, -8, and -9 in spontaneous neutrophil apoptosis [14, 22–26]. The CaspaTag™ method was used to follow the activation of these three caspases in cultured neutrophils. Increased activity of caspase-3, -8, and -9 was already detected at 6 h in untreated neutrophils (**Fig. 5**). The addition of fumonisin B_2 to the culture medium clearly reduced caspase activation at this

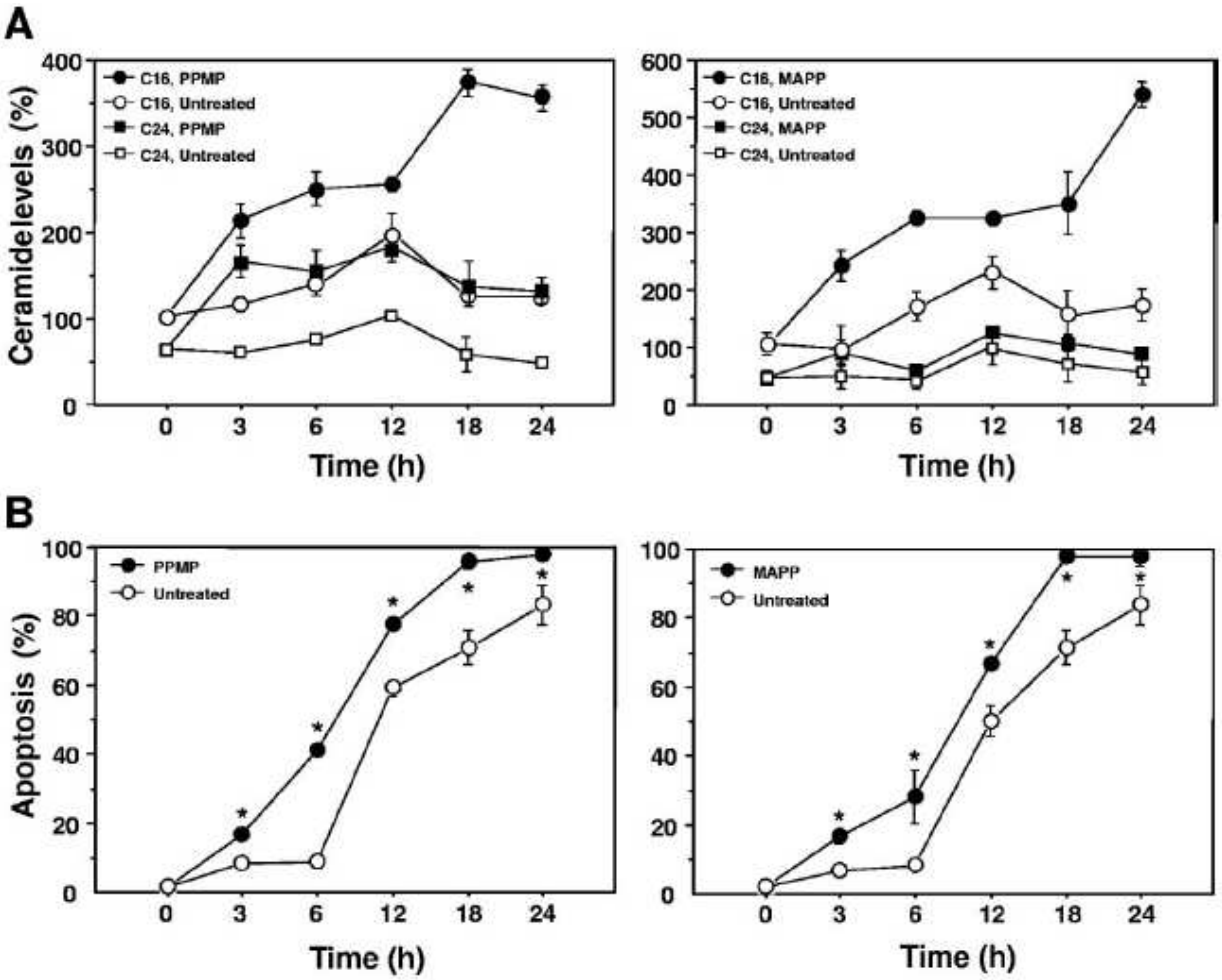

Fig. 3. Treatment of cultured blood neutrophils with PPMP and MAPP. (A) Blood neutrophils were incubated in the absence (Untreated) or presence of 30 μM PPMP or 50 μM MAPP. After 0, 3, 6, 12, 18, and 24 h incubation, C_{16}- and C_{24}-ceramide levels were measured using LC-ESI-MS/MS. (B) Untreated and treated neutrophils were assayed for apoptosis at the different time-points using dual-color Annexin-V-FITC/PI staining and flow cytometry analyses. *, Significantly different from the results obtained with untreated cells ($P<0.05$).

time-point (Fig. 5). Conversely, treatment with MAPP and PPMP significantly increased caspase activity at 6 h (Fig. 5). Together, these results indicate that de novo-generated C_{16}- and C_{24}-ceramides act upstream of caspase-3, -8, and -9 in neutrophils.

GM-CSF prevents C_{16}- and C_{24}-ceramide accumulation in neutrophils

Numerous inflammatory mediators are able to promote neutrophil survival in vitro and in vivo [27]. Among these molecules, GM-CSF crucially contributes to inhibition of neutrophil apoptosis at the site of inflammation [28]. Evidence for such an antiapoptotic effect of GM-CSF is provided in **Figure 6A**, where it can be seen that apoptosis of GM-CSF-treated neutrophils is delayed significantly as compared with that of untreated controls. Figure 6B shows that treatment of cultured neutrophils with GM-CSF totally prevented accumulation of de novo-generated C_{16}- and C_{24}-ceramides, a finding that could partly explain the antiapoptotic function of this cytokine.

Fas ligation accelerates neutrophil apoptosis without affecting de novo ceramide generation

Resting neutrophils respond to Fas engagement by accelerating their apoptosis rate [29], a finding that we confirmed in the present study (**Fig. 7A**). Indeed, a threefold increase in apoptosis was observed at 6 h in sFasL-treated neutrophils when compared with untreated controls. At 12, 18, and 24 h, the differences in apoptosis between sFasL-treated and untreated neutrophils were less pronounced but still significant. Figure 7B shows that treatment with sFasL did not alter the levels of C_{16}- and C_{24}-ceramides in neutrophils, indicating that sFasL-induced acceleration of neutrophil apoptosis is not dependent on a rise in de novo ceramide production.

DISCUSSION

Blood neutrophils enter apoptosis spontaneously and are removed from the circulation by macrophages within 24–48 h of

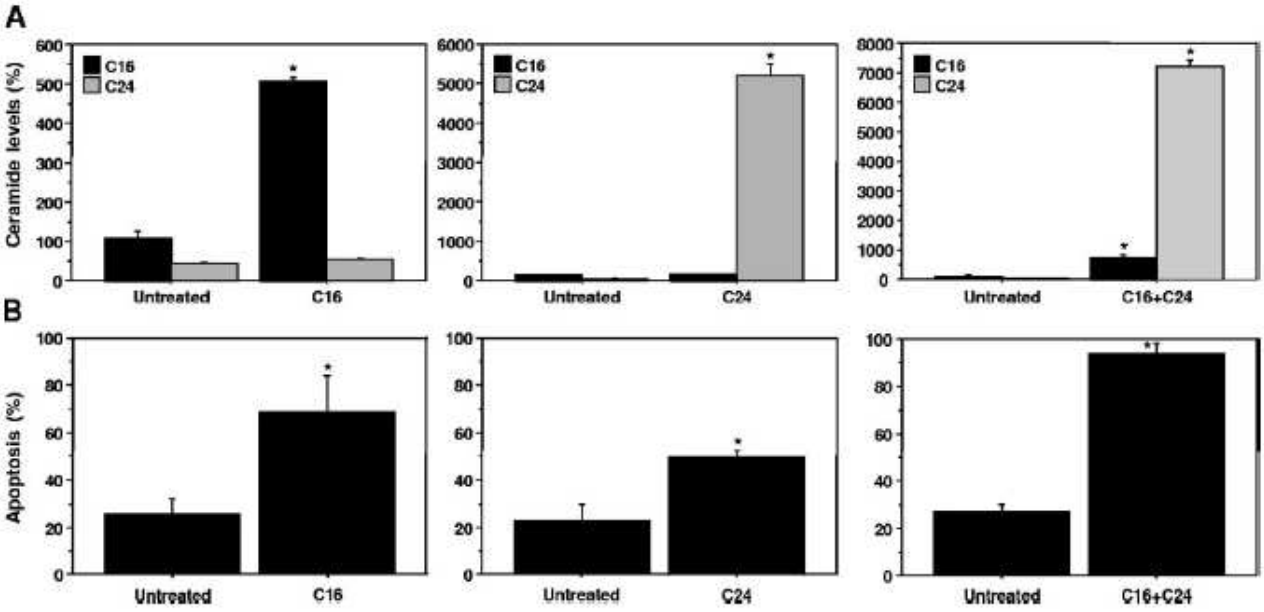

Fig. 4. Effects of treatment with synthetic C_{16}- and C_{24}-ceramides on intracellular ceramide contents and apoptosis rates. (A) Freshly isolated blood neutrophils were treated with 100 μM synthetic C_{16}-ceramide (left panel) or 100 μM synthetic C_{24}-ceramides (middle panel). Alternatively, neutrophils were treated with a combination of 100 μM synthetic C_{16}-ceramide and 100 μM synthetic C_{24}-ceramides (right panel). Six hours after treatment, intracellular C_{16}- and C_{24}-ceramide levels were assessed by LC-ESI-MS/MS. Untreated cells served as controls. *, Significantly different from the values obtained with untreated cells ($P<0.05$). (B) Six hours after treatment with exogenous C_{16}- and/or C_{24}-ceramides, apoptosis was evaluated by staining with Annexin-V-FITC and PI (left panel for treatment with exogenous C_{16}-ceramide, middle panel for treatment with exogenous C_{24}-ceramide, and right panel for treatment with both cermaide species). *, Significantly different from the results obtained with untreated cells ($P<0.05$).

leaving the bone marrow [1, 2]. Therefore, the neutrophil turnover is rapid, minimizing the risk of loss of their toxic contents to the surrounding tissues. Although central to homeostasis, the mechanisms that control neutrophil apoptosis remain elusive. In this study, we have addressed the question of what is the exact role of ceramides in regulating neutrophil lifespan. Ceramides are lipid second messengers involved in many antiproliferative responses such as cell-cycle arrest, senescence, and apoptosis [10, 11]. Previous reports have implicated ceramides in neutrophil physiology. First, lactosylceramide-enriched glycosphingolipid signaling microdomains mediate superoxide generation in neutrophils through the PI-3K-, p38 MAPK-, and protein kinase C-dependent signal transduction pathway [30]. Second, apoptosis may be induced in neutrophils through ROS-induced ASMase activation and subsequent ceramide accumulation [14]. In our study, treatment with fumonisin B_2, a selective ceramide synthase inhibitor [18, 19], not only blocked C_{16}- and C_{24}-ceramide accumulation but also reduced apoptosis in neutrophils. Conversely, 3-OMS and fantofarone, which are specific inhibitors of NSMase and ASMase, respectively, neither affected intracellular ceramide levels nor prevented apoptosis in these cells. Our results therefore provide evidence that de novo-generated C_{16}- and C_{24}-ceramides play a role in spontaneous neutrophil apoptosis. C_{16}- and C_{24}-ceramide accumulation was inhibited completely by fumonisin B_2 and therefore appeared to be exclusively dependent on the de novo synthesis pathway. However, fumonisin B_2 only slowed down neutrophil apoptosis, indicating that de novo C_{16}- and C_{24}-ceramides play a role but are not absolutely required for the apoptotic process in these cells and that other signaling mechanisms, independent of ceramide biosynthesis, are involved in induction of spontaneous neutrophil death. Prominent among these mechanisms appears to be the gradual loss of Mcl-1, an antiapoptotic protein of the Bcl-2 family, in aging neutrophils [31]. More recently, Zhu et al. [32] have demonstrated that deactivation of the phosphatidylinositol 3,4,5-trisphosphate/Akt signaling pathway also plays a key role in induction of neutrophil spontaneous death.

Under normal conditions of de novo sphingolipid synthesis, ceramides are not considered to be end-products with biological activities, as they serve as substrates for sphyngomyelin synthase and glucosylceramide synthase, enzymes of complex sphingolipid synthesis. However, there is now a growing body of evidence to implicate de novo-generated ceramides in induction of apoptosis [11]. For example, consistent with our findings, accumulation of de novo C_{16}- and/or C_{24}-ceramides provokes apoptosis in lymphoma cells following BCR stimulation [33], in LNCaP prostate cancer cells after androgen ablation [34], and in dendritic cells (DC) cultured in the presence of tumor supernatants [35].

An intriguing question that remains is: What are the molecular mechanisms that boost the de novo ceramide synthesis pathway, thereby leading to accumulation of C_{16}- and C_{24}-ceramides and eventually to apoptosis in neutrophils? It has been demonstrated that the de novo pathway may be activated in response to TNF-α [36], free palmitoyl-CoA [37], lymphotoxin [38], BCR cross-linking [33], and chemotherapeutic

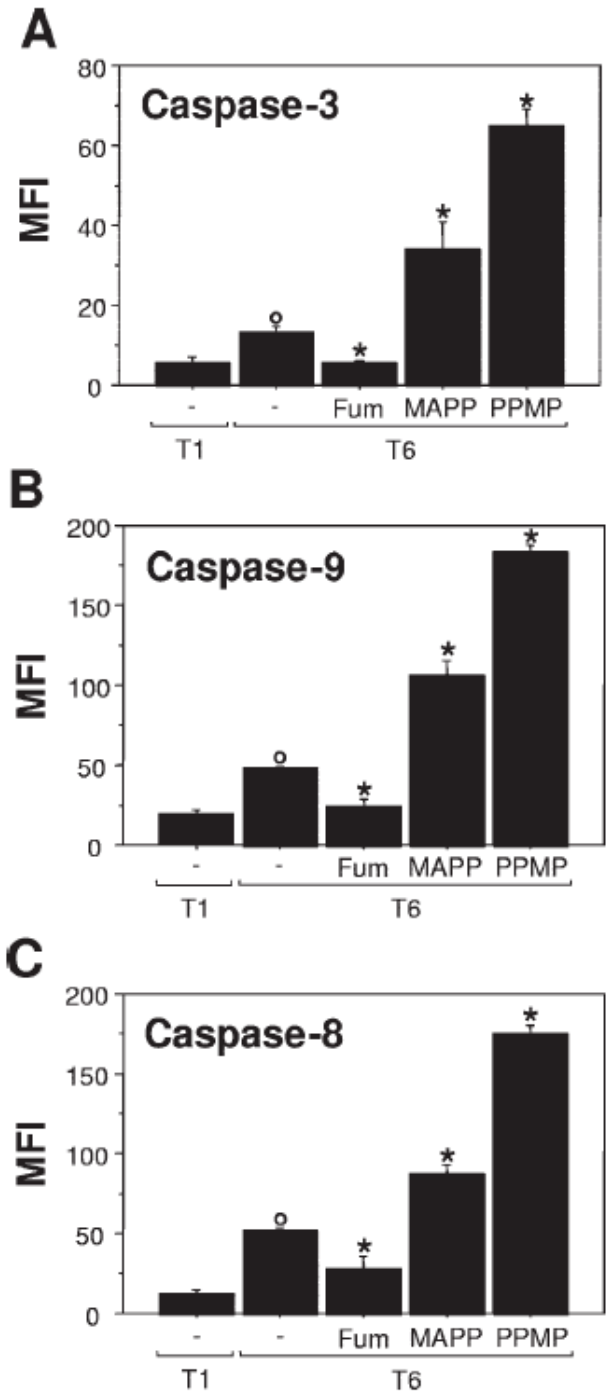

Fig. 5. Activity of caspase-3, -8, and -9 in untreated and fumonisin B_2-, MAPP-, and PPMP-treated neutrophils. Blood neutrophils were isolated and cultured for 1 h (T1) or 6 h (T6) in the absence or presence of 50 μM fumonisin B_2 (Fum), 50 μM MAPP, or 30 μM PPMP. Caspase-3, -8, and -9 activity was measured using the CaspaTag™ method and flow cytometry analyses. MFI, Mean fluorescence intensity. °, Significantly different from the results obtained at T1 ($P<0.05$); *, significantly different from the results obtained with untreated cells ($P<0.05$).

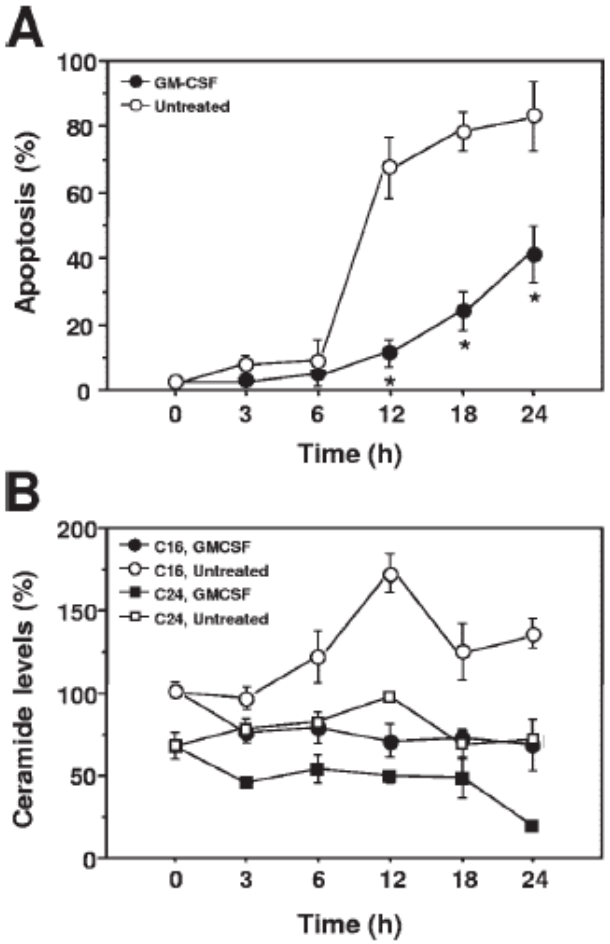

Fig. 6. Effects of GM-CSF on apoptosis and C_{16}- and C_{24}-ceramide levels. (A) Blood neutrophils were treated with GM-CSF (125 U/ml) for 0, 3, 6, 12, 18, and 24 h before apoptosis was assessed by Annexin-V-FITC/PI staining and flow cytometry analyses. Untreated cells were used as controls. *, Significantly different from the results obtained with untreated cells ($P<0.05$). (B) Intracellular C_{16}- and C_{24}-ceramide levels were measured at the different time-points using LC-ESI-MS/MS.

agents such as daunorubicin [39], hexadecylphosphocholine [40], and etoposide [41]. In our study, neutrophils were left untreated, indicating that cell autonomous mechanisms are involved in the activation of the de novo pathway. Further investigations are needed to decipher these mechanisms.

The use of LC-MS to identify specific molecular species of ceramide has revealed the selective formation of C_{16}- and C_{24}-ceramide during operation of the de novo pathway, indicating that those ceramides are a potential signature for this pathway [11]. Accumulation of de novo-generated C_{16}-ceramide has been reported to be responsible for apoptosis induction in various cell types. For example, BCR-induced apoptosis of lymphoma cells involves a highly selective increase in the levels of C_{16}-ceramide, formed specifically through the de novo pathway [32]. Moreover, Eto et al. [34] showed that increases in C_{16}-ceramide levels generated via the de novo pathway play a key role during the apoptosis induced by androgen ablation in the androgen-dependent LNCaP prostate

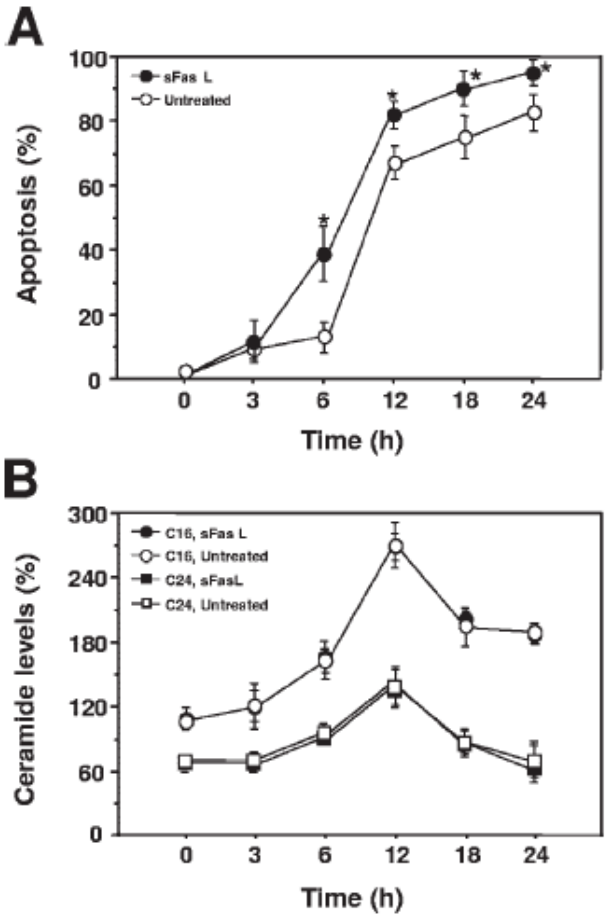

Fig. 7. Effects of treatment with sFasL on apoptosis and C_{16}- and C_{24}-ceramide levels. (A) Blood neutrophils were treated with sFasL (25 ng/ml) for 0, 3, 6, 12, 18, and 24 h before apoptosis was assessed by Annexin-V-FITC/PI staining and flow cytometry analyses. Untreated cells were used as controls. *, Significantly different from the results obtained with untreated cells ($P<0.05$). (B) Intracellular C_{16}- and C_{24}-ceramide levels were measured at the different time-points using LC-ESI-MS/MS.

cancer cell line. Here, we provide several lines of evidence extending the role of de novo-generated C_{16}-ceramide to the initiation of spontaneous neutrophil apoptosis. First, C_{16}-ceramide was by far the predominant ceramide species present in neutrophils and was much more abundant in neutrophils than in any of the other cell types investigated. Second, fumonisin B_2 totally abrogated C_{16}-ceramide accumulation and substantially reduced apoptosis in cultured neutrophils. Finally, exogenously supplied C_{16}-ceramide was particularly potent in inducing neutrophil death. Our data, however, also implicate a participation of de novo-generated C_{24}-ceramide in spontaneous neutrophil apoptosis. Indeed, C_{24}-ceramide rates increased significantly before apoptosis detection in cultured neutrophils, a process that was inhibited by fumonisin B_2. Moreover, synthetic C_{24}-ceramide, although less potent than C_{16}-ceramide, was able to induce neutrophil death. Finally, exogenously added C_{16}- and C_{24}-ceramides had an additive effect on neutrophil apoptosis. Our findings are therefore consistent with a previous report that de novo-generated C_{24}-ceramide, in combination with de novo-synthesized C_{16}-ceramide, participates in tumor-induced DC apoptosis [35].

Once generated, ceramides can be phosphorylated by ceramide kinase to yield ceramide-1-phosphate or can serve as substrates for the synthesis of sphingomyelin or glucosylceramides. Alternatively, ceramide can be metabolized by ceramidases to yield sphingosine. In this study, we have used PPMP (namely, a selective inhibitor of glucosylceramide synthase) and MAPP (namely, a specific ceramidase inhibitor) to block ceramide metabolism and to demonstrate definitively that accumulation of ceramides may trigger the apoptotic program in neutrophils. PPMP increased C_{16}- and C_{24}-ceramide levels significantly in cultured neutrophils, whereas MAPP only enhanced C_{16}-ceramide concentrations in these cells, indicating that C_{16}-ceramide is converted into glucosylceramide or sphingosine, and C_{24}-ceramide is only transformed into glucosylceramide. These findings show that the different ceramide species can be processed through distinct metabolic pathways in neutrophils.

Activation of caspase-3, -8, and -9 plays a crucial role in the induction and execution of spontaneous neutrophil apoptosis [14, 22–26]. We have therefore examined whether de novo-generated ceramides participate in caspase activation in neutrophils. It has been reported that short-chain ceramides can induce poly(ADP-ribose) polymerase proteolysis and caspase activation, two key events in the apoptotic process [42, 43]. Moreover, ceramides produced by the action of sphingomyelinases are also potent inducers of caspase activity [44]. The first studies to examine the role of de novo-generated ceramides in apoptosis were by Perry et al. [41]. Their results provided evidence that ceramides from the de novo synthesis pathway and caspases activate an independent pathway during etoposide-induced apoptosis of Molt-4 leukemia cells. In their model, de novo-synthesized ceramides induce caspase-independent cell death by effecting changes in the membrane structure, whereas caspases provoke DNA fragmentation in a ceramide-independent manner. Consistent with this model, tumor-induced DC apoptosis requires de novo formation of C_{16}- and C_{24}-ceramides but occurs via a caspase-independent pathway [35]. However, other studies placed de novo-generated ceramides upstream of caspase activation in the apoptotic program. Indeed, caspase activation and subsequent apoptosis are consecutive to de novo ceramide production in IgM-stimulated lymphoma cells and androgen-deprived LNCaP prostate cancer cells [33, 34]. In the present study, treatment of neutrophils with fumonisin B_2 substantially reduced activation of caspase-3, -8, and -9, whereas treatment with MAPP and PPMP enhanced caspase activity, demonstrating that de novo-generated ceramides act upstream of caspases in these cells. Moreover, as caspase-8 activation leads to the cleavage of Bid in neutrophils and therefore precedes mitochondrial depolarization in these cells [14], we also conclude that de novo generation of C_{16}- and C_{24}-ceramides is an early event (upstream of mitochondrial depolarization) in spontaneous neutrophil apoptosis.

Neutrophils have a short half-life, which is extended at the inflammatory site by a range of inflammatory mediators and bacterial components. Among these factors, GM-CSF potently delays neutrophil apoptosis in vivo [28]. The molecular mech-

anisms by which GM-CSF controls neutrophil apoptosis are not fully understood. It has been demonstrated that the protective effects of GM-CSF involve tyrosine kinase activity and subsequent activation of MAPKs [45]. In addition, it has been shown that GM-CSF-delayed apoptosis in neutrophils is associated with a cooperative regulation of Mcl-1, an antiapoptotic protein of the Bcl-2 family, by the Janus kinase/STAT and PI-3K pathways [46]. In parallel, it has been reported that the survival effect of GM-CSF is caused by a PI-3K-dependent phosphorylation and cytosolic translocation of Bad, a proapoptotic member of the Bcl-2 family [47]. In this study, we show that GM-CSF totally abrogates accumulation of de novo-generated C_{16}- and C_{24}-ceramides, thereby providing a new mechanism by which GM-CSF may prevent neutrophil apoptosis. However, as GM-CSF had a greater effect on inhibiting neutrophil apoptosis than did fumonisin B_2 (compare Fig. 2B, left panel, with Fig. 6A), it is evident that inhibition of C_{16}- and C_{24}-ceramide production is not the sole mechanism accounting for the antiapoptotic effects of GM-CSF. Further studies are underway to clarify the importance of inhibition of de novo ceramide synthesis in GM-CSF-mediated antiapoptosis.

Stimulation of the Fas death pathway results in rapid apoptosis in neutrophils [29]. We therefore sought to determine whether de novo-generated ceramides participate in Fas-induced neutrophil apoptosis. Fas engagement triggers de novo ceramide production and apoptosis in Jurkat cells [48]. However, although de novo-generated ceramides are implicated in regulating the dephosphorylation of serine/arginine-rich proteins in response to Fas activation in Jurkat cells [49], it appears that de novo-produced ceramides do not act as critical mediators of Fas-induced apoptosis in these cells [48]. In the present study, treatment of neutrophils with sFasL accelerated apoptosis but did not modify the levels of C_{16}- and C_{24}-ceramides, indicating that Fas-induced neutrophil apoptosis does not require an increase in de novo ceramide generation.

In conclusion, we show that de novo-generated C_{16}- and C_{24}-ceramides contribute to spontaneous neutrophil apoptosis by acting upstream of caspase-3 activation. Moreover, we provide evidence that GM-CSF exerts its antiapoptotic effects on neutrophils, at least partly through inhibition of de novo ceramide accumulation.

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