Significant Role of Ceramide Pathway in Experimental Gastric Ulcer Formation in Rats

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ABSTRACT

Ceramides have emerged as key participants in the signaling pathway of cytokines and apoptosis. We previously revealed that phorbol 12-myristate 13-acetate (PMA) induced experimental ulcers in rat gastric mucosa. In this study, we investigated the role of ceramide in ulcer formation and its relation to the activation of transcription factors and apoptosis. PMA was suberosally injected to rat glandular stomach. Fumonisins B1 (FB1), an inhibitor of ceramide synthase, was administered together with the PMA. The time course of ceramide content was quantified using thin layer chromatography and the number of apoptotic cells was determined by immunohistochemistry. The activation of transcription factor nuclear factor-κB (NF-κB) or activator protein-1 (AP-1) was evaluated using an electrophoretic mobility shift assay. The administration of FB1 attenuated PMA-induced gastric ulcer formation in a dose-dependent manner. Before the ulcers became obvious, the ceramide content (C18 and C24 ceramide) increased significantly in the gastric wall. The activation of NF-κB and AP-1 and an increase in the number of apoptotic cells were also observed. Both of these were significantly inhibited by the coadministration of FB1. However, NF-κB inhibitors attenuated gastric ulcer formation without affecting the ceramide content or the number of apoptotic cells. Ceramide formation in the stomach significantly contributes to PMA-induced tissue damage, possibly via the activation of transcription factors and an increase in apoptosis in the gastric mucosa. However, after the increase in ceramide levels, the NF-κB and apoptosis pathways may be separable involved in ulcer formation.

Sphingolipids are derivatives of a number of common chemical backbones known as long-chain (sphingoid) bases (Spiegel and Merrill, 1996). These lipids seem to be ubiquitous among eukaryotic organisms and have long been recognized as having roles in membrane structure. Recent discoveries have revealed that sphingolipids (e.g., ceramide, sphingosine) are highly bioactive compounds that are involved in diverse cell processes, including cell-cell interactions, adhesion, cell proliferation, differentiation, and oncogenic transformation (Hakomori and Igarashi, 1995). In particular, ceramide has received attention as an important bioactive molecule that may participate in the mediation of some of the actions of extracellular agents, such as tumor necrosis factor-α (TNF-α) (Dbaibo et al., 1993; Tepper et al., 1995) and interferon-γ (Kim et al., 1991). Ceramide has also been reported to induce IL-6 production in fibroblasts (Laulederkind et al., 1995), IL-2 secretion in lymphocytes (Mathias et al., 1993), and IL-1-induced E selectin expression in endothelial cells (Masamune et al., 1996), suggesting that ceramide is involved in the regulation of immune function and inflammatory responses.

Several stimuli that induce the generation of ceramide also activate transcription factor nuclear factor-κB (NF-κB). The activation of NF-κB involves the proteasomal degradation of its cytoplasmic inhibitors IκBs (Verma et al., 1993; Baldwin, 1996). This pathway allows the translocation of free, active NF-κB complexes into the nucleus, where they bind to cognate DNA sequences in the promoter/enhancer regions of a large number of target genes involved in immune responses and inflammation. However, the role of ceramide in NF-κB activation remains controversial. In several reports, ceramide did not induce the transcription of NF-κB-dependent

ABBREVIATIONS: TNF-α, tumor necrosis factor-α; IL, interleukin; NF-κB, nuclear factor-κB; PMA, phorbol 12-myristate 13-acetate; FB1, fumonisins B1; PHTLC, high-performance thin layer chromatography; AP-1, activator protein-1; EMSA, electrophoretic mobility shift assay; FITC, fluorescein isothiocyanate; ss, single-stranded; TBS, Tris-buffered saline.
genes (Westwick et al., 1995; Latinis and Koretzky, 1996). In others, the ceramide response could not be dissociated from NF-κB activation (Dhaibo et al., 1993; Betts et al., 1994; Higuchi et al., 1996). Nevertheless, arguments in favor of a role for ceramide in NF-κB activation also exist. In several cell lines, including HL-60 leukemia cells, Jurkat cells, and human umbilical vein endothelial cells, the activation of NF-κB by ceramide has been demonstrated (Schutze et al., 1992; Yang et al., 1993; Masamune et al., 1996). Masamune et al. (1999) recently described the formation of NF-κB-specific DNA-protein complexes consisting of nuclear proteins from Kato III cells (a gastric cancer cell line) that had been treated with C2-ceramide (Masamune et al., 1999). However, no information has been reported on the interaction between ceramide and NF-κB activation in the gastric mucosa.

Ceramide has also been suggested to have a role in signaling apoptosis induced by the addition of extracellular agents, such as TNF-α (Dhaibo et al., 1993; Kolesnicky and Golde, 1994) or the anti-Fas antibody (Cifone et al., 1994), and analogs of ceramide have been reported to induce apoptosis (Obeid et al., 1993; Jarvis et al., 1994; Sweeney et al., 1996). In addition to its acute proinflammatory potency, TNF-α has been shown to have a direct cytotoxic effect on gastric epithelial cells (Fiorucci et al., 1998). However, the relationship between ceramide formation and the induction of apoptosis and gastric mucosal damage during the process of gastric ulcer formation has not been investigated.

We previously reported that the subserosal injection of phorbol 12-myristate 13-acetate (PMA) resulted in the formation of gastric ulcers in the rat gastric mucosa (Takeuchi et al., 2002). In that study, activation of NF-κB in the gastric mucosa corresponding to the PMA injection sites was observed, and the ulcer formation was significantly inhibited by the inhibitors of NF-κB or an antibody against TNF-α, suggesting that both NF-κB activation and the subsequent TNF-α release may contribute to tissue damage in PMA-induced gastric ulcer formation. Using an experimental model, the aims of this study were to 1) investigate whether the activation of ceramide occurs in the gastric mucosa and is involved in the induction of apoptosis and experimental ulcer formation, and 2) determine whether ceramide formation influences the activation of transcription factors, including NF-κB, in the process of gastric ulcer formations.

Materials and Methods

Animals and Ulcer Induction. Male Wistar rats, weighing 200 to 250 g and maintained on standard laboratory chow (Oriental Yeast Mfg., Ltd., Tokyo, Japan), were used in all the experiments. All animals were handled according to the guidelines of Keio University School of Medicine, Animal Research Committee. The rats were not given any food for 24 h before the experiments but were allowed access to tap water ad libitum. Under anesthesia with 30 mg/kg pentobarbital sodium, the abdomen was opened with a midline incision. The stomach was exposed, and 50 μl of either PMA (Sigma-Aldrich, St. Louis, MO) (at a dose of 50 μg) or its vehicle (20% ethanol in saline) was injected into the subserosal layer of the anterior wall of the glandular stomach using a microsyringe. The abdomen was then closed.

At different time intervals (between 0.5 and 48 h) after the injection of PMA or its vehicle, the rats were killed using an overdose of sodium pentobarbital (500 mg/kg). Their stomachs were rapidly removed, opened along the greater curvature, and rinsed with cold normal saline. The surface area of each lesion in the gastric mucosa was assessed macroscopically. Mucosal injury was also histologically evaluated using paraffin sections stained with hematoxylin and eosin and scored using a 0 to 4 scale based on the following criteria: 0, normal; 1, patches of superficial necrosis; 2, vascular congestion and focal necrosis of less than one-third of the mucosa; 3, vascular congestion and focal necrosis of more than one-third of the mucosa, but not reaching the full thickness; and 4, extensive vascular congestion and necrosis involving the full thickness of the mucosa.

**Administration of Various Inhibitors.** To examine ceramide activation in this model, an inhibitor of sphingolipid biosynthesis, 0.01 to 10 μM fumonisin B1 (FB1) (10 μl of 0.05–50 μM FB1 equals 0.5–500 pmol) (Sigma-Aldrich), was coadministered along with the PMA. To determine the role of NF-κB in PMA-induced ulcer formation, 100 nM pyrrolidine dithiocarbamate (PDTC) (10 μl of 500 mM PDTC equals 5 pmol) (Sigma-Aldrich) and an NF-κB decoy (15 mM) (10 μl of 75 μM decoy of NF-κB equals 0.75 μmol) were locally injected into the stomach in combination with the PMA. The sequence of the synthetic double-stranded oligodeoxynucleotides (NF-κB decoy) was as follows: 5′-CTCTGAGGGATTTCTCCTC-3′ and 3′-GGAACTTTCCCTAAAGGAGG-5′.

We chose these NF-κB inhibitor concentrations because there is evidence that these doses sufficiently inhibited the activation of NF-κB as well as ulcer formation in our previous experiments (Takeuchi et al., 2002). To assess the apoptotic effects in PMA-induced ulcer formation, 10 to 100 nM Z-DEVD-FMK, a caspase 3/7 inhibitor (10 μl of 50–500 nM caspase 3/7 inhibitor equals 0.5–5 pmol) (Calbiochem, San Diego, CA) and 10 to 100 nM Ac-Tyr-Val-Ala-Asp-H, a caspase 4/1 inhibitor (10 μl of 50–500 nM caspase 4/1 inhibitor equals 0.5–5 pmol) (Peptide Institute, Osaka, Japan), were also locally injected into the stomach in combination with the PMA.

**Determination of Ceramide Content in the Stomach.** The time course of the ceramide content in the stomach was quantified. The excised stomachs were cut along the greater curvature and rinsed with physiological saline. Approximately 0.5 g of the tissue sample, including the ulcer lesions, was removed and minced, and lipid extraction was performed by a modified version of the method described by Bligh and Dyer (Berg et al., 1997). After the extraction of the major lipids, the neutral lipids, including the ceramides, were separated using high-performance thin layer chromatography (HPTLC) (Silicagel 60; Merck, Darmstadt, Germany) (Kasama et al., 1996). The dried lipids were then resolved by thin layer chromatography using petroleum ether and diethyl ether (7:3) as the first solvent and chloroform and methanol (95:5) as the second solvent.

After lipid separation, the HPTLC plate was sprayed with a primulin reagent until thoroughly wet and then air-dried completely. The lipids were visualized under UV light at 365 nm and analyzed with a densitometer (Fluorochrom 8000; Alpha Innotech Corporation, San Leandro, CA).

**Activation of NF-κB and AP-1.** The activation of NF-κB and AP-1 was assessed in the PMA-injected gastric mucosa using an electrophoretic mobility shift assay (EMSA). The stomachs were excised under pentobarbital anesthesia at different time intervals (between 15 min and 3 h) after the PMA administration. Glandular stomach was homogenized on ice in 3.5 μl of buffer containing 10 mM HEPES (pH 7.9); 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM dithiothreitol; 0.5 mM phenylmethylsulfonyl fluoride; and 2 μg/ml each of antipain, chymostatin, bestatin, pepstatin, and leupeptin. The lysate was centrifuged at 8000g for 2 min at 4°C, and the cytoplasmic supernatant was removed and frozen. The nuclear pellet was reconstituted in 1.5 μl of buffer containing 20 mM HEPES (pH 7.9); 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride; and 2 μg/ml each of antipain, chymostatin, bestatin, pepstatin, and leupeptin, followed by vigorous vortexing for 20 min at 4°C. The nuclear lysates were centrifuged at 14,000g for 5 min, and the nuclear extracts were divided into aliquots, frozen, and stored at −80°C. A gel shift assay was performed using a fluorescein isothiocyanate (FITC)-labeled NF-κB probe.
synthetic double-stranded oligonucleotide (AGTTGAGGGGACTTTCCCAGGC) and an AP-1 synthetic double-stranded oligonucleotide (CCAAAGTGCTGAGTCACTAAT). Equal amounts of nuclear proteins (20 μg) were incubated for 30 min at room temperature with 1 pg of labeled NF-κB and AP-1 consensus oligonucleotide. After incubation, the samples were electrophoresed through nondenaturing 6% polyacrylamide gels at 4°C, 160 V for 2 h. For the competition analysis, a 100-fold molar excess of unlabeled consensus oligonucleotide was added to the samples. The fluorescence intensity of the gels was quantified using a fluorescence laser scanning system equipped with a computer-assisted image analyzer (FluorImager 575; Amersham Biosciences Inc., Sunnyvale, CA).

**Determination of Apoptosis in the Gastric Mucosa.** Apoptosis in the stomach after PMA administration was immunohistochemically examined using a polyclonal antibody to ss-DNA (Korkolopoulou et al., 2001). The area of the stomach containing the ulcer was rapidly excised and processed using routine techniques before being embedded in paraffin. Sections (4 μm in thickness) were then prepared and mounted on glass slides. Deparaffinized sections were treated with 3% hydrogen peroxide for 20 min to block endogenous peroxide. After blocking in 10% nonimmune serum for 10 min at room temperature, sections were incubated for 40 min at room temperature with primary antibody (anti-ss-DNA, polyclonal rabbit; DAKO, Carpinteria, CA) diluted 1:100 with 0.1% bovine serum albumin in 0.05 M Tris-buffered saline (TBS). The slides were washed three times with 0.05 M TBS-Tween for 5 min followed by incubation for 30 min with rabbit peroxidase (DAKO). After being washed for 5 min in TBS-Tween, the sections were stained using a diaminobenzidine reagent set (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and observed by microscopy (ECLIPSE-E-600; Nikon, Tokyo, Japan). Negative controls containing nonimmune rabbit serum or the omission of the primary antibody was also prepared. Staining for all antibodies was assessed in a blind manner by the same observer.

**Statistical Analysis.** All results were expressed as the means ± S.E.M. Differences among groups were evaluated using a one-way analysis of variance and Fisher’s post hoc test. The histological damage scores were analyzed by Mann-Whitney’s U test. Statistical significance was set at $p < 0.05$.

**Results**

Figure 1A shows representative pictures of a PMA-induced gastric mucosal lesion 48 h after the subserosal injection of PMA and the inhibitory effect of different concentrations of FB1 (0.1 and 10 μM) on ulcer formation. The ulcers produced by the PMA injection arose at the injection site in the stomach, but were significantly inhibited by FB1; a fumonisin dose of 10 μM almost completely blocked the PMA-induced ulcer formation. Figure 1B shows the area of the mucosal lesions and the histological damage scores. The size of ulcers and histological damage induced by PMA were dose dependently inhibited by the fumonisin treatment. FB1 doses of 0.1 μM or more significantly attenuated the area of the mucosal lesions and the histological damage score.
Figure 2A shows the time course for the changes in C18 and C24 ceramide contents in gastric mucosal lesions after the injection of 50 μg of PMA. In the rat gastric mucosa, the main ceramides induced by PMA were C18 and C24. Figure 2B shows a quantitative analysis of the changes in C18 (left) and C24 (right) ceramide contents. The amounts of both ceramides significantly increased 3 h after PMA injection and reached their maximal values at 3 to 6 h. Thereafter, the ceramide contents began to decrease, but remained at elevated levels, compared with the controls at 48 h.

Figure 3A shows the inhibitory effect of FB1 (10 μM) and NF-κB inhibitors (PDTC and NF-κB decoy) on ceramide activation 3 h after PMA administration, whereas Fig. 3B shows the quantitative analysis of the changes in C18 (left) and C24 (right) ceramide contents caused by these treatments. The activation of both ceramides in response to PMA was significantly attenuated by FB1. However, these activations were not significantly inhibited by the NF-κB inhibitors, PDTC, or the NF-κB decoy.

Figure 4 shows the effect of FB1 on the activation of NF-κB and AP-1 in the gastric mucosa 3 h after the injection of PMA, as determined by EMSA using FITC-labeled NF-κB and an AP-1 consensus oligonucleotide. PMA treatment induced a significant increase in NF-κB, and AP-1 binding activity compared with the vehicle treatment (control). The increase in NF-κB binding activity at 3 h after PMA administration was significantly attenuated by treatment with FB1 (0.01–10 μM). The increase in AP-1 binding activity at 3 h after PMA administration was also dose dependently attenuated by treatment with FB1. Although not shown in this figure, the activation of NF-κB binding activity was also inhibited by the NF-κB inhibitors, PDTC, and the NF-κB decoy.

Figure 5 shows the effect of caspase inhibitors on PMA-induced gastric ulcer formation and the histological damage scores. Caspase 3/7 and caspase 1/4 inhibitors both attenuated the PMA-induced gastric mucosal lesions and histological damage scores. Caspase 3/7 and caspase 1/4 inhibitors both attenuated the PMA-induced gastric mucosal lesions and histological damage scores. Caspase 3/7 and caspase 1/4 inhibitors both attenuated the PMA-induced gastric mucosal lesions and histological damage scores. Caspase 3/7 and caspase 1/4 inhibitors both attenuated the PMA-induced gastric mucosal lesions and histological damage scores.

Figure 6 presents representative microscopic findings showing apoptotic cells in the gastric mucosa after PMA administration, as determined by immunohistochemistry using a polyclonal antibody against ss-DNA. In control ethanol-treated mucosa specimens, only a few apoptotic cells were observed in the gastric mucosa. In contrast, a few apoptotic cells were observed at the tip of the mucosa 0.5 h after PMA treatment, and their number significantly increased at 3 h after treatment. Six hours after treatment, the mucosal surface has been destroyed and the diffuse pattern of apoptotic cells was observed in the deeper layers of the stomach. After 24 h, the distribution of these apoptotic cells extended deeply toward the submucosa in the area corresponding to the lesion and in its vicinity.

Figure 7 shows the time course for the changes in the number of apoptotic cells in the gastric mucosa after PMA administration, as immunohistochemically assessed using microscopy, and the effects of FB1, NF-κB inhibitors, and caspase 3/7 and caspase 1/4 inhibitors. A significant increase in apoptotic cells was observed at 3 h after administration, reaching maximum value at 24 h after administration. Thereafter, the number of apoptotic cells began to decrease somewhat with the development of mucosal defects. FB1, the caspase 3/7 inhibitor, and the caspase 1/4 inhibitor significantly inhibited the PMA-induced increase in the number of apoptotic cells at 24 h after administration (Figs. 6F and 7). In contrast, neither PDTC nor the NF-κB decoy significantly attenuated the increase in the number of apoptotic cells after PMA administration, although in our previous article we demonstrated that the same concentrations of PDTC or NF-κB significantly attenuated the ulcer area and tissue damage (Takeuchi et al., 2002).

Discussion

Fumonisins are a family of mycotoxins that are produced by some strains of Fusarium moniliforme (Sydenham et al., 1999). The mycotoxin is produced by Fusarium moniliforme, which is a common fungus found in many plant materials. Fumonisins cause a variety of health problems in animals and humans, including polyps and tumors in various organs. The mycotoxin is produced by the fungus and is toxic to animals and humans. The mycotoxin is produced by the fungus and is toxic to animals and humans. The mycotoxin is produced by the fungus and is toxic to animals and humans.
FB1 is a potent inhibitor of sphinganine (sphingosine) N-acetyltransferase (ceramide synthase) in vivo, and it exhibits competitive-type inhibition with respect to both substrates of this enzyme (Wang et al., 1991). Our present results showing that the blockade of ceramide synthase by FB1 successfully attenuates experimentally induced gastric ulcer formation suggest the importance of de novo ceramide synthesis in this process. Ceramide synthase is responsible for the acylation of sphinganine in the de novo biosynthetic pathway for sphingolipids as well as the reutilization of sphingosine derived from sphingolipid turnover (Wang et al., 1991; Merrill et al., 1993). Although ceramide derived from the hydrolysis of sphingomyelin in response to extracellular signals seems to be important in most pathways (Ballou et al., 1996; Hannun, 1996), ceramide production from de novo synthesis via ceramide synthase may also largely account for its bioactive roles as a messenger in the stimulation of a variety of cellular functions. Ohta et al. (1995) demonstrated that HL-60 cells treated with PMA exhibited an increase in both ceramide and sphingosine, which agrees with our present results. In the case of PMA-injected gastric mucosa, the excess production of ceramide via ceramide synthase plays a critical role in the development of gastric tissue damage, although the exact downstream processes remain unknown.

In a previous study, we presented data indicating that the activation of NF-κB in the gastric mucosa after PMA injection generally corresponded to the location of the gastric gland cells (Takeuchi et al., 2002). In that study, we also revealed the critical role of NF-κB activation in the formation of PMA-induced gastric ulcer. The present study shows that
the activation of NF-κB and AP-1 induced by PMA was significantly inhibited by treatment with FB1, suggesting that ceramide synthesis is involved in the activation of these transcription factors. Johns et al. (1994) demonstrated that the ceramide pathway additionally activates proteins that bind to an AP-1 consensus site, suggesting that the ceramide pathway may serve more globally to induce genes that are responsive to both NF-κB and AP-1 (Johns et al., 1994).

Because we previously found that PMA-induced acute gastric ulcer formation is mediated by the strong activation of NF-κB in situ, the blocking effect of fumonisin B1 may arise from significant attenuation in transcription factors, especially NF-κB. Phorbol esters, such as PMA, stimulate protein kinase C by mimicking the effects of diacylglycerol and also induce NF-κB activation. Fernandez and Dobbelaeere (1999) showed that ceramide and PMA have a synergistic effect on the degradation of IκB in primary lymph node T cells as well as in transformed T cells. Thus, ceramide may potentiate NF-κB activation induced in PMA-injected gastric mucosa. The activation of NF-κB leads to the activation of various proinflammatory molecules, including TNF-α, IL-2, IL-6, inducible nitric-oxide synthase (Jourd’heuil et al., 1997), and adhesion molecules responsible for leukocyte-endothelial interactions (Dhawan et al., 1997). These inflammatory mediators could in turn activate NF-κB, initiating a vicious inflammatory cycle that is likely to lead to tissue damage (Barnes and Karin, 1997).
In the present study, we clearly demonstrated that the number of apoptotic cells was increased 3 h after the injection of PMA. This increase in apoptotic cells initially occurred mainly in the upper part of the glandular mucosa and thereafter in the deeper layer at 6 h, and extended deeply toward the submucosa at 24 h. The increase in apoptotic cells in the gastric mucosa was similar to a previously reported time course for ulcer formation (Takeuchi et al., 2002). In addition, both caspase 3/7 and caspase 1/4 inhibitors significantly attenuated PMA-induced ulcer formation in this study. This suggests that apoptosis is involved in the formation of gastric ulcers. The increase in apoptosis in the gastric mucosa corresponded with the increase in C18 and C24 ceramide content in the stomach wall. We also observed a significant reduction in PMA-induced apoptosis in the gastric mucosa after treatment with FB1. Ceramide is known to be involved in signaling apoptosis, and ceramide analogs have been reported to induce apoptosis in vitro using gastric mucosal cell lines (Johal and Hanson, 2000; Shimada et al., 2000). These findings suggest that the ceramide pathway may be strongly associated with the increase in apoptotic cell death in the PMA-injected mucosa, although the involvement of other pathways, including interleukin-1-converting enzyme-like proteases, cannot be ruled out (Sweeney et al., 1996). However, it should also be noted that the inhibition of NF-κB activation did not successfully prevent the increase in apoptosis in this ulcer model. Even though ceramide can activate NF-κB in the gastric mucosa and NF-κB activation contributes to PMA-induced ulcer formation, the NF-κB and apoptosis pathways may be separately involved in the ulcer formation (Fig. 8). Further investigation of the downstream signals in ceramide activation is necessary to elucidate the exact role of this molecule in the formation of gastric ulcers.

References


Fig. 8. Schematic diagram illustrating the pathways involved in PMA-induced ceramide formation and the steps experimentally performed in this study.


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