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ABSTRACT

Monocytes are peripheral leukocytes that function in innate immunity. Excessive triglyceride (TG) accumulation causes monocyte death and thus can compromise innate immunity. However, the mechanisms by which TG mediates monocyte death remain unclear to date. Thus, this study aimed to elucidate the mechanisms by which TG induces monocyte death. Results showed that TG induced monocyte death by activating caspase-3/7 and promoting poly(ADP-ribose)polymerase (PARP) cleavage. In addition, TG induced DNA damage and activated the ataxia telangiectasia mutated (ATM)/checkpoint kinase 2 and ATM-and Rad3-related (ATR)/checkpoint kinase 1 pathways, leading to the cell death. Furthermore, TG-induced DNA damage and monocyte death were mediated by caspase-2 and -8, and caspase-8 acted as an upstream molecule of caspase-2. Taken together, these results suggest that TG-induced monocyte death is mediated via the caspase-8/caspase-2/DNA damage/executioner caspase/PARP pathways.

INTRODUCTION

Triglycerides (TG), nonpolar lipid molecules consisting of three fatty acid molecules esterified to glycerol, are the main constituent of dietary fat and lipid storage in the human body (1). During the postprandial period, TG-rich lipoproteins (TRL) are synthesized as very-low-density lipoprotein and chylomicrons, being transported into the systemic circulation, where they can interact with circulating immune cells (2). Hyperlipidemia, characterized by elevated serum total cholesterol and/or TG levels, compromises innate immunity and proper immune response to bacterial challenges, thereby increasing susceptibility to infections (3). Monocytes are innate immune compartments that play crucial roles in recognizing foreign pathogens and eliminating invading pathogens. This immune reaction is accompanied by the production of pro-inflammatory cytokines and chemokines as well as the proliferation of monocytes (4). Postprandial TRL can cause apoptosis and cell cycle arrest in THP-1 cells, an established human monocyte line (5), implying that hypertriglyceridemia can impair innate immune function partly through TG-mediated monocytopenia. However, the signaling pathways involved in monocytes have not been extensively studied.

DNA damage, caused by endogenous reactive oxygen species generation, environmental genotoxin exposure, and heavy metal exposure (6), triggers programmed cell death to maintain genomic integrity. The DNA damage response signaling pathway is activated by ataxia-telangiectasia mutated (ATM) and/or ataxia telangiectasia and Rad3-related (ATR), resulting in G2/M cell cycle arrest and DNA repair by nonhomologous end joining or apoptosis under irreparable DNA damage (7, 8). A previous clinical study reported that DNA damage occurs in human lymphocytes isolated from patients with hyperlipidemia (9). However, whether DNA damage occurs in monocytes isolated from patients with hyperlipidemia has not yet been determined.

Caspase-2 is an initiator caspase that is activated by certain cellular stresses, such as heat shock and cytoskeletal disruption (10). Activated caspase-2 cleaves Bid, a pro-apoptotic Bcl-2 family protein, which consequently induces cytochrome C release from the mitochondria, resulting in caspase-9-mediated activation of executioner caspases and apoptosis (7, 10, 11). In addition, caspase-2 is involved in DNA damage-induced cell death (12). Caspase-8 is another initiator caspase whose activity is triggered by stimulation of a death receptor, such as Fas, to participate in extrinsic and intrinsic apoptotic pathways (13). It activates caspase-activated DNase (CAD) under sub-lethal levels of extrinsic stimuli, inducing DNA damage (14). Boege et al. reported that caspase-8 can sense DNA damage by assembling the caspase-8/ receptor Interacting Protein Kinase-1 (RIPK1)/fas associated protein with a death domain (FADD)/ cellular FLICE-like inhibitory protein (c-FLIP) complex (15).

This study aimed to explore the signaling pathways involved in TG-induced monocyte death. We revealed the involvement of caspase-8, caspase-2, ATM, ATR, caspase-3/7, and PARP in TG-induced monocyte death, providing information to understand the link between hypertriglyceridemia and monocytopenia.

RESULTS

TG reduces the viability of THP-1 monocytes by inducing apoptosis

Consistent with our previous report, we found in the present study that TG can decrease the viability of THP-1 monocytes (16). As shown in Fig. 1A, TG reduced the viability of THP-1 monocytes in a dose- and time-dependent manner. Then, the TG-treated THP-1 monocytes were stained with propidium iodide and subjected to flow cytometry to determine whether the TG-induced decrease in cell viability is due to proliferation suppression or cell death. The cell cycle of THP-1 monocytes was unaffected by TG treatment (Supplementary Fig. 1), implying that the TG-induced reduction in cell viability was not due to proliferation suppression. However, western blot results showed that the level of cleaved PARP increased in a TG dose- and time-dependent manner (Fig. 1B). In addition, the cleavage/activation of caspase-3 and caspase-7, which are upstream molecules of PARP, increased in a TG dose- and time-dependent manner (Fig. 1C). These results suggest that TG induces caspase-dependent cell death in TG-treated monocytes.

TG induces DNA damage causing cell death in THP-1 monocytes

Caspase-dependent cell death can be induced by various stimuli, such as DNA damage (17-19). DNA damage is observed in polymorphonuclear leukocytes isolated from patients with hyperlipidemia (9, 20). Therefore, we performed a comet assay to examine whether DNA damage is implicated in the TG-induced monocyte death. As shown in Fig. 2A, unlike the untreated cells, the TG-treated THP-1 monocytes displayed classical comet tails, indicative of DNA damage. In addition, the phosphorylation of H2AX, another marker of DNA damage, increased in a TG dose- and time-dependent manner in the TG-treated THP-1 monocytes (Fig. 2B). Furthermore, the ATM-Chk2 and ATR-Chk1 pathways, which are two distinct kinase signaling cascades activated in response to DNA damage (21, 22), were activated in the TG-

treated THP-1 monocytes (Fig. 2B). Then, THP-1 monocytes were incubated with TG in the absence or presence of CGK733, a specific inhibitor of the ATM/ATR pathway, for 24 h to determine whether DNA damage is involved in the TG-induced death of these monocytes. Treatment with CGK733 restored the TG-induced cleavage of PARP in an inhibitor dose-dependent manner (Fig. 2C), and the TG-induced decreased viability of THP-1 monocytes was recovered using CGK733 treatment (Fig. 2D). These results indicate that TG induces DNA damage, which consequently stimulates caspase-dependent cell death in TG-treated THP-1 monocytes.

Caspase-2 is implicated in the TG-induced death of THP-1 monocytes

Caspase-2 is activated by various forms of cellular damage, including DNA damage, and is associated with stress-induced cell death by inducing mitochondrial membrane permeabilization and executioner caspase activation (7, 11, 12, 23). Therefore, we investigated whether caspase-2 is involved in the TG-induced death of THP-1 monocytes. The result showed that caspase-2 activity increased in the TG-treated THP-1 monocytes in a time-dependent manner (Fig. 3A) and that caspase-2 inhibition significantly rescued TG-induced DNA damage (Fig. 3B). These results indicated that caspase-2 was involved in the TG-induced DNA damage in the THP-1 monocytes. In addition, the TG-induced phosphorylation of ATM, Chk2, and H2AX was suppressed by caspase-2 inhibition in a dose-dependent manner (Fig. 3C). However, caspase-2 inhibition did not affect the phosphorylation of ATR and Chk1 in the TG-treated THP-1 monocytes (Fig. 3C). In addition, suppression of caspase-2 activity reduced the TG-induced cleavage of PARP (Fig. 3D) and restored the viability of the TG-treated THP-1 monocytes (Fig. 3E). Taken together, these results suggest that the TG-induced death of THP-1 monocytes is mediated by the activation of caspase-2, which induces DNA damage.

Caspase-8 is involved in TG-induced monocyte death

Caspase-8 induces cell death via cytochrome C release from the mitochondria (24, 25). Caspase-8 regulates caspase-2 activity in the saikosaponin A-induced apoptosis of human colon carcinoma cell lines (26). Therefore, we examined whether caspase-8 is involved in the TG-induced death of THP-1 monocytes. Results showed that TG treatment increased caspase-8 cleavage in a dose- and time-dependent manner (Fig. 4A), indicating that caspase-8 is activated in TG-treated monocytes. Then, we investigated the relationship between caspase-2 and caspase-8 in the TG-induced death of THP-1 monocytes. As shown in Fig. 4B, caspase-8 inhibition significantly blocked the TG-induced activation of caspase-2. By contrast, caspase-2 inhibition did not affect the TG-induced cleavage of caspase-8, indicating that caspase-8 is an upstream molecule of caspase-2 in TG-induced monocyte death. Moreover, caspase-8 inhibition suppressed the TG-induced phosphorylation of ATM, ATR, Chk1, and Chk2 (Fig. 4C), implying that caspase-8 is associated with TG-induced DNA damage. Caspase-8 inhibition decreased TG-induced PARP cleavage (Fig. 4D) and recovered the viability of TG-treated monocytes in an inhibitor dose-dependent manner (Fig. 4E). These results suggest that TG-induced monocyte death is mediated by caspase-8, caspase-2, DNA damage, and executioner caspases (Supplementary Fig. 2).

DISCUSSION

Hyperlipidemia contributes to genomic instability. Cholesterol treatment promotes phosphorylated H2AX foci formation inside the nucleus in human umbilical vein endothelial cells (27). An animal experiment using an acute hyperlipidemia Wistar rat model demonstrated that DNA damage occurs in various tissues, such as the liver, kidney, and peripheral blood (28). Similarly, the thoracic aortas of high-fat diet-fed *ApoE*^{-/-} mice show higher phosphorylated H2AX levels than those of normal diet-fed mice, implying the involvement of hyperlipidemia in DNA damage (29). In the present study, treatment with TG induced DNA damage in monocytes, suggesting that DNA damage in monocytes can be presented in patients with hyperlipidemia, which subsequently causes monocyte death. The resultant monocytopenia can compromise innate immunity.

Caspases can induce not only apoptosis but also DNA damage. Activated caspase-8 enhances caspase-3 activation, which cleaves inhibitor of CAD and subsequently activates CAD in neuronal cells during ischemia (30). This event results in apoptotic cell death or DNA damage accumulation without apoptosis. The present study also showed that caspase-8 was associated with TG-induced DNA damage in THP-1 monocytes. Caspase-8 may be involved in CAD activation in TG-treated THP-1 monocytes, subsequently resulting in DNA damage. Meanwhile, caspase-2 can contribute to genomic stability through its non-apoptotic role. A recent study has reported that caspase-2 deficiency leads to DNA fork stalling and S-phase arrest in mouse embryonic fibroblasts and that sustained S-phase can induce stalled or collapsed replication fork, resulting in DNA double-strand breakage (31). By contrast, our study showed that caspase-2 activation was associated with DNA damage in the TG-treated THP-1 monocytes. To the best of our knowledge, the current study is the first to demonstrate that caspase-2 mediates DNA damage. Further studies using cell lines and/or triggers other than TG are needed to investigate the role of caspase-2 in DNA damage.

In addition, caspase-2 inhibition blocked ATM activation in the present study. ATM is an upstream molecule of caspase-2 in irradiated cervical cancer cell lines (7, 8, 32). Caspase-2 possibly acts not only as an effector molecule after DNA damage in an irradiated cervical cancer cell line but also as a trigger molecule of DNA damage in monocytes under hypertriglyceridemia. Therefore, caspase-2 seems to act differently in DNA damage-associated processes depending on the type of cell and/or trigger.

A previous study reported that treatment with postprandial TRL causes apoptosis and S-phase accumulation in THP-1 monocytes (5), whereas the current study showed that the cell cycle was unaffected by TG treatment (Supplementary Fig. 1). With the different lipid components between the TG emulsion used in this study and postprandial TRL, cell cycle arrest can be mediated by either non-TG components or the synergistic effect of TG and other lipids.

We previously reported that TG triggers caspase-1- and caspase-2-mediated cell death in phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 macrophages (33, 34). We found that activated caspase-2 is also involved in TG-mediated THP-1 monocyte death. By contrast, caspase-1 did not contribute to the death of TG-treated THP-1 monocytes (Supplementary Fig. 3). These results suggest that the TG responses of THP-1 monocytes and PMA-differentiated THP-1 macrophages are cell type-specific.

Taken together, this study revealed the cellular signaling pathways related to TG-induced monocyte death, which may serve as a reference for the development of drugs to alleviate TG-induced monocytopenia and/or compromised innate immunity.

MATERIALS AND METHODS

Materials

TG emulsion Lipofundin® MCT/LCT 20% (B. Braun Melsungen AG, Melsungen, Germany) was used to deliver TG into cells as described previously (35). Caspase-1 substrate Ac-YVAD-p-nitroanilide (Ac-YVAD-pNA) was purchased from Biomol (Plymouth Meeting, PA, USA). Caspase-2 substrate Ac-VAVAD-pNA and RNase A were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against cleaved caspase-8, cleaved caspase-3, cleaved caspase-7, PARP, p-ATM, p-ATR, p-Chk1, p-Chk2, and p-H2AX were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against β -actin and the caspase-2-specific inhibitor z-VDVAD-FMK were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The caspase-8-specific inhibitor z-IETD-FMK was obtained from R&D Systems (Minneapolis, MN, USA). The ATM/ATR kinase inhibitor CGK733 was purchased from Calbiochem (Darmstadt, Germany).

Cell culture

The human acute monocytic leukemia cell line THP-1 was purchased from ATCC (Manassas, VA, USA) and cultured in RPMI 1640 medium supplemented with heat-inactivated 10% fetal bovine serum and penicillin–streptomycin. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂, seeded at a density of 1×10^6 cells per well in six-well plates, and then treated with TG in the absence or presence of individual inhibitors for 24 h.

Cell viability test

The trypan blue dye exclusion assay was used to enumerate viable cells as previously described (36). Briefly, 0.4% trypan blue staining solution (10 μ L) was mixed with the trypsinized cell suspension (10 μ L). Non-stained cells were counted using a hemocytometer

(Marienfeld, LaudaKönigshofen, Germany).

Measurement of caspase activity

Caspase-2 were measured as previously described (37). THP-1 cells were washed and lysed with phosphate-buffered saline (PBS) containing 1% Triton X-100 and then centrifuged at $19,000 \times g$ for 10 min at 4 °C. The supernatant was collected, and the protein concentration was quantified. Caspase-2 activity was measured by combining protein with the caspase-2 substrate Ac-VAVAD-pNA (200 μ M) in 150 μ L of PBS. The reactions were incubated for 3 h at 37 °C, and the activity was determined by obtaining the absorbance at 405 nm.

Western blot analysis

THP-1 cells were washed with PBS and lysed at 4 °C in PBS containing 1% Triton X-100, protease inhibitor cocktail (Sigma-Aldrich), phosphatase inhibitor cocktail (Roche, Mannheim, Germany), and PBS. The lysates were clarified, and the supernatants were subjected to western blot as previously described (38).

Comet assay

The comet assay was performed using a comet assay kit (Trevigen, Gaithersburg, MD, USA). Cells were centrifuged, washed with ice-cold PBS, resuspended in a 0.5% (w/v) solution of low-temperature melting agarose in PBS at 37 °C, and then layered onto comet slides (Trevigen). The agarose was incubated at 4 °C for 30 min and then placed in a lysis solution containing 1% sodium lauryl sarcosinate, 10 mM Tris base, 2.5 mM NaCl, 100 mM EDTA, and 0.01% Triton X 100 (Trevigen) for 30 min at 4 °C in the dark. The slide was subsequently immersed in alkaline unwinding solution containing 1 mM EDTA and 200 mM NaOH. Electrophoresis was performed in an alkaline buffer at 1 V/cm for 25 min at 4 °C, and the gel

was washed twice in distilled water. The washed slides were dried at 45 °C for 20 min and then stained with SYBR Green (Trevigen). Images were obtained using a laser confocal scanning microscope (LSM 710; Zeiss, Heidenheim, Germany).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). *p*-values were calculated using Student's *t*-tests, and differences were considered to be statistically significant at $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$. Data are presented as mean \pm standard error of the mean (SEM). Each experiment was conducted at least thrice.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FIGURE LEGENDS**Figure 1. TG induces the death of THP-1 monocytes**

(A) THP-1 monocytes were treated with the indicated concentration of TG for the indicated times, and viable cells were enumerated by trypan blue dye exclusion assay. The number of viable THP-1 monocytes incubated for 24 h without TG treatment was set as 100%. *p*-values were determined with Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. THP-1 monocytes were incubated with the indicated concentration of TG for 24 h or with TG (1.0 mg/mL) for the indicated times. Western blot was performed with (B) anti-PARP antibody and (C) anti-cleaved caspase-3 and anti-cleaved caspase-7 antibodies. β -actin was used as an internal control.

Figure 2. TG induces DNA damage in THP-1 monocytes

(A) THP-1 monocytes were treated with or without TG for 24 h, and comet assays were performed to assess DNA damage. (B) THP-1 monocytes were incubated with the indicated concentration of TG for 24 h or with TG (1.0 mg/mL) for the indicated times. Phosphorylation of H2AX, Chk1, Chk2, ATR, and ATM was detected using western blot. THP-1 monocytes were treated with TG (1.0 mg/mL) in the absence or presence of the ATM/ATR inhibitor CGK733 for 24 h. (C) PARP cleavage was detected using western blot, and (D) viable cells were enumerated using trypan blue dye exclusion assay. The number of viable THP-1 monocytes without TG and inhibitor treatment was set as 100%. *p*-values were determined with Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. Caspase-2 is involved in TG-induced DNA damage leading to THP-1 monocyte death

(A) Caspase-2 activity was assessed in THP-1 monocytes treated with TG (1.0 mg/mL) for the

indicated times. The absorbance of THP-1 cells without TG treatment was set as 1.0. **(B)** THP-1 monocytes were incubated with TG (1.0 mg/mL) in the absence or presence of the caspase-2 inhibitor z-VDVAD-FMK (10 μ M) for 24 h. Comet assays were performed to assess DNA damage. THP-1 monocytes were treated with TG (1.0 mg/mL) in the absence or presence of z-VDVAD-FMK for 24 h. **(C)** Phosphorylation of H2AX, Chk1, Chk2, ATR, and ATM and **(D)** cleavage of PARP were detected using western blot. **(E)** THP-1 monocytes were incubated with TG (1.0 mg/mL) in the absence or presence of z-VDVAD-FMK (10 μ M) for 24 h. Viable cells were enumerated using trypan blue dye exclusion assay. The number of viable cells in THP-1 monocytes without TG and inhibitor treatment was set as 100%. *p*-values were determined with Student's *t*-test. * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001.

Figure 4. Caspase-8 is an upstream molecule of caspase-2 in TG-induced THP-1 monocyte death

(A) THP-1 monocytes were incubated with the indicated concentration of TG for 24 h or with TG (1.0 mg/mL) for the indicated times, and cleavage of caspase-8 was detected using western blot. **(B)** Caspase-2 activity was assessed in THP-1 monocytes treated with TG (1.0 mg/mL) in the absence or presence of the caspase-8 inhibitor z-IETD-FMK (10 μ M) for 24 h. The absorbance of THP-1 cells without TG and caspase-8 inhibitor treatment was set as 1.0 (left). THP-1 monocytes were incubated with TG (1.0 mg/mL) in the absence or presence of the caspase-2 inhibitor z-VDVAD-FMK (10 μ M) for 24 h and cleavage of caspase-8 was detected using western blot (right). **(C, D)** THP-1 monocytes were treated with TG (1.0 mg/mL) in the absence or presence of z-IETD-FMK for 24 h. **(C)** Phosphorylation of ATR, ATM, Chk1, and Chk2 and **(D)** cleavage of PARP were detected using western blot. **(E)** THP-1 monocytes were treated with TG (1.0 mg/mL) without or with z-IETD-FMK for 24 h. Viable cells were enumerated using trypan blue dye exclusion assay. The number of viable THP-1 monocytes

without TG and inhibitor treatment was set as 100%. p -values were determined with Student's t -test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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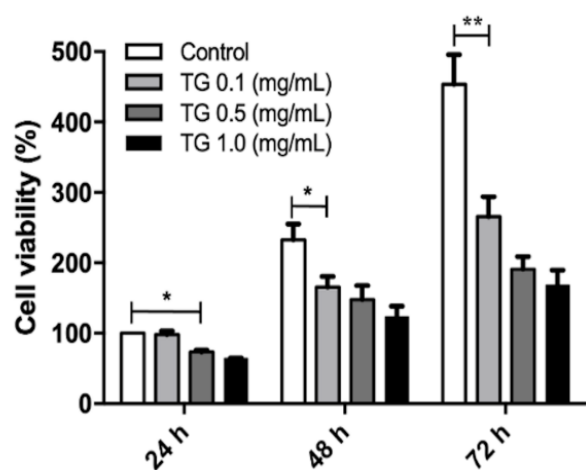
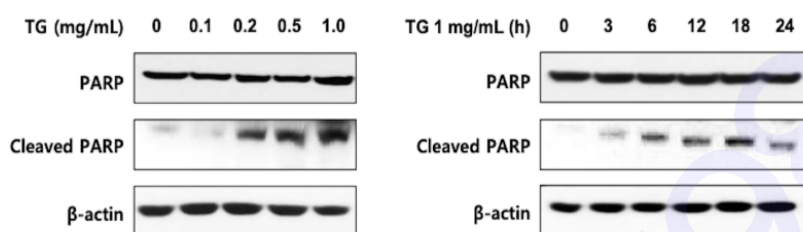
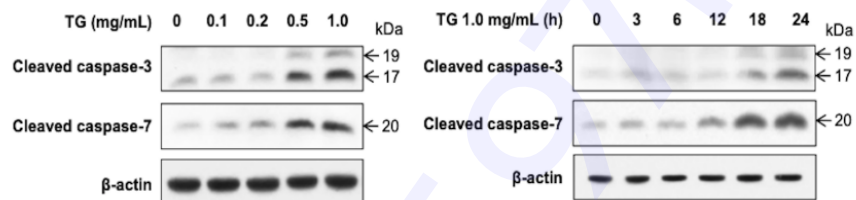
A**B****C**

Fig. 1.

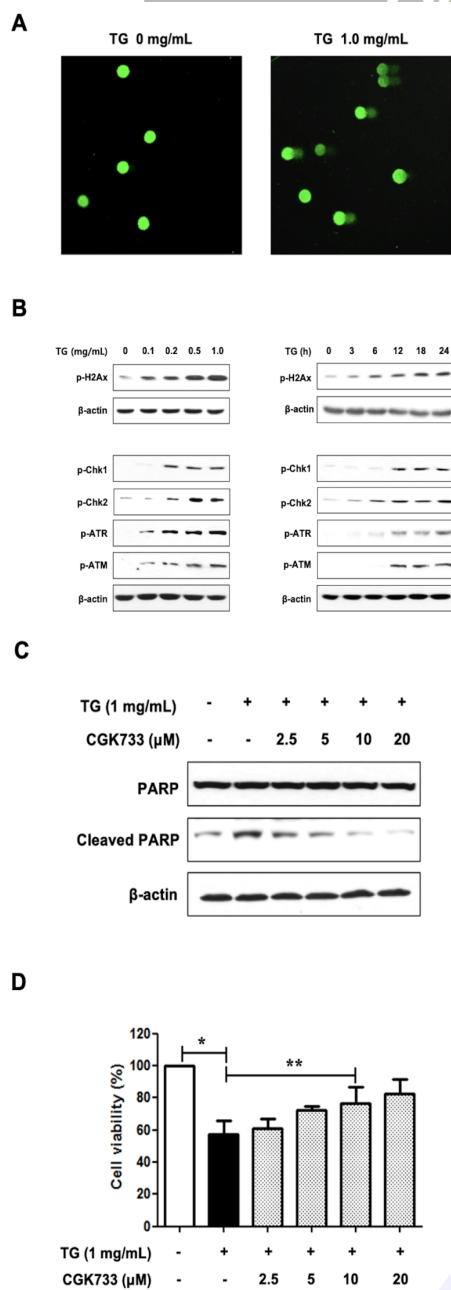
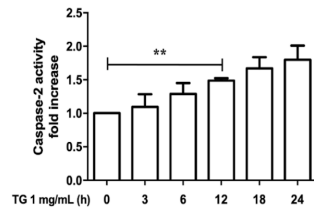
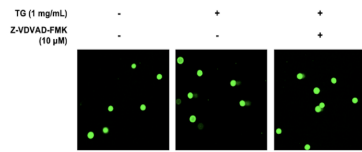


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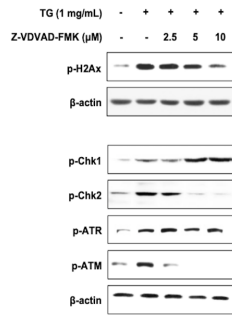
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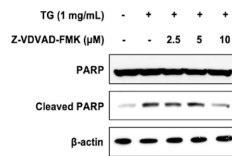
B



C



D



E

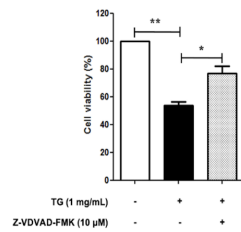
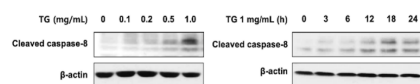
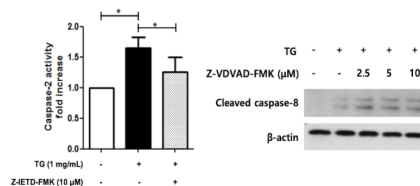


Fig. 3.

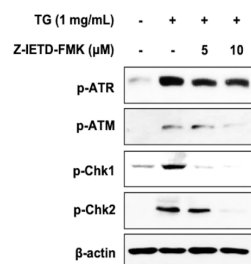
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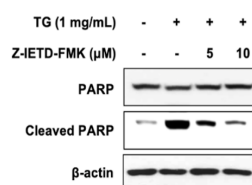
B



C



D



E

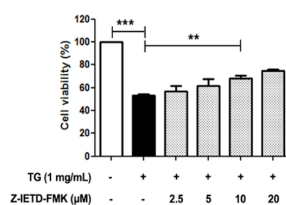


Fig. 4.

Supplementary Data**Triglyceride induces DNA damage leading to monocyte death by activating caspase-2 and caspase-8**

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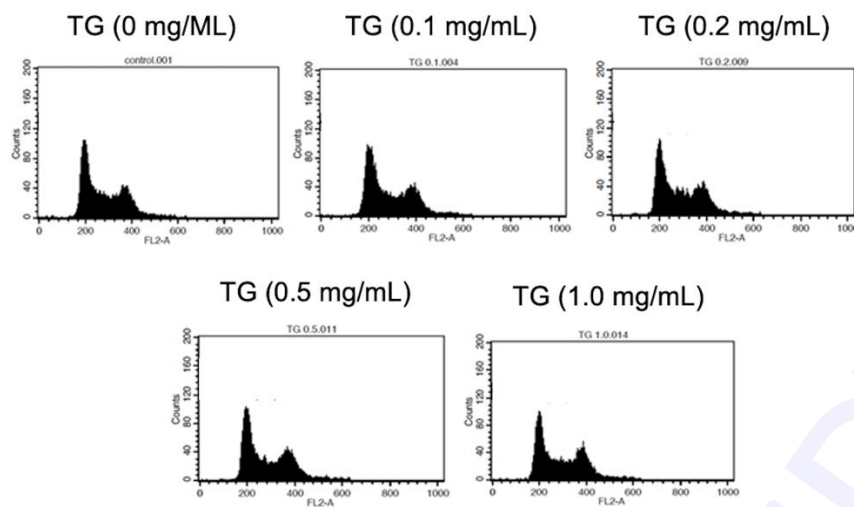
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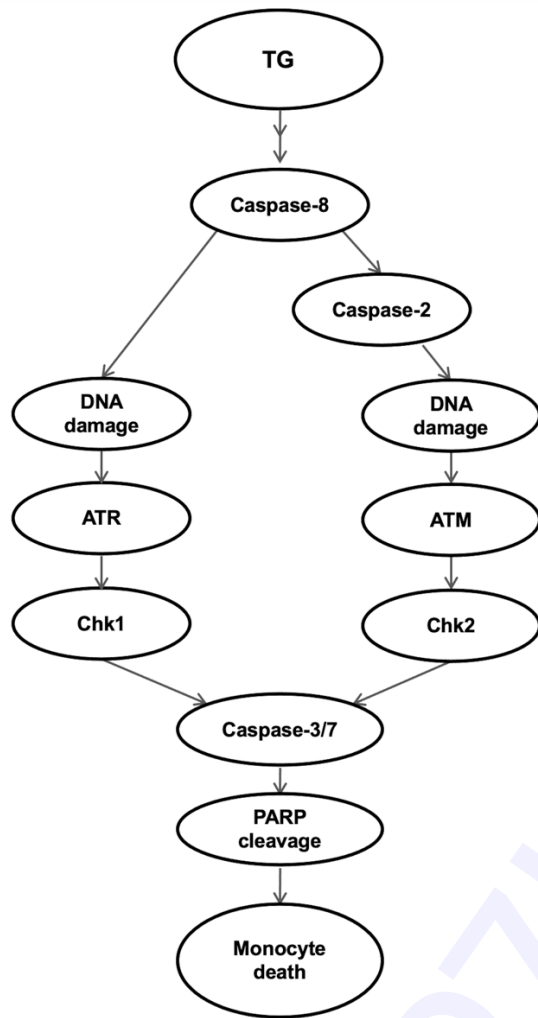
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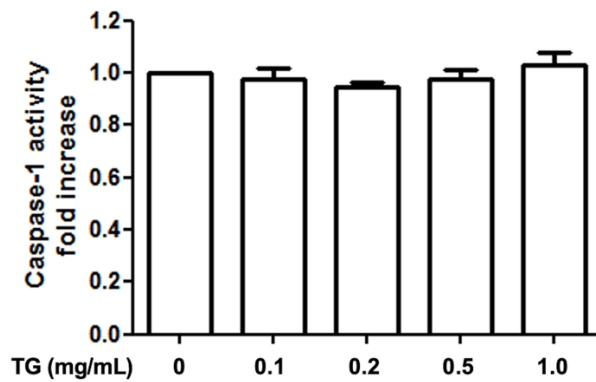
Tel : 82-41-570-4149, E-mail : sunghoonkim@kornu.ac.kr



Supplementary Fig. 1. DNA content of THP-1 monocytes was measured using flow cytometry. DNA contents of THP-1 monocytes were measured using flow cytometry. THP-1 monocytes were seeded in 6-well plates at a density of 2×10^5 cells/well and were cultured in 10% FBS-RPMI 1640 media with indicated concentrations of TG for 24 h. Next, after centrifugation, the cells were washed twice with phosphate-buffered saline (PBS). After centrifugation for 5 minutes at $100 \times g$ at 4°C , the THP-1 cells were resuspended in prechilled 70% ethanol and stored for 2 hours at 4°C . After centrifugation, the cells were washed with PBS. The fixed cells were stained with a PBS solution containing 5 mg/mL of PI and 0.1 mg/mL RNase A. After incubation for 40 minutes at 37°C in the dark, the cell suspension was analyzed by FACS Calibur (BD biosciences).



Supplementary Fig. 2. Schematic representation of the signal pathway through which TG induces DNA damage and cell death of monocytes.



Supplementary Fig. 3. Caspase-1 activity was assessed in THP-1 monocytes treated with the indicated concentration (0, 0.1, 0.2, 0.5, or 1.0 mg/mL) of TG for 24h. THP-1 cells were washed with PBS and lysed with phosphate-buffered saline (PBS) buffer containing 1% Triton X-100 and then centrifuged at 19,000 x g for 10 minutes at 4°C. The supernatant was collected and the protein concentration was quantified. To measure caspase-1 activity, protein (90 µg) was combined with caspase-1 substrate, Ac-YVAD-pNA (200 µM) in 150 µL of PBS. Reactions were incubated for 3 h at 37°C, and the activity was determined by measuring the absorbance at 405 nm. The absorbance of THP-1 cells without TG treatment was set as 1.0.