Triglyceride-Induced Macrophage Cell Death Is Triggered by Caspase-1

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Triglyceride (TG) induces macrophage cell death which contributes to the development of atherosclerosis. We confirmed that exogenous TG accumulates in human THP-1 macrophages and causes cell death. TG treated THP-1 macrophages exhibited no change in tumor necrosis factor (TNF)-α, interleukin (IL)-18, macrophage inflammatory protein (MIP)-1α, and IL-1RI receptor mRNA expression. However, there was a marked decrease in IL-1β mRNA expression but an increase in IL-1β protein secretion. Decreased expression of IL-1/β mRNA and increased secretion of IL-1/β protein was not the direct cause of cell death. Until now, TG was assumed to induce necrotic cell death in macrophages. Since caspase-1 is known to be involved in activation and secretion of IL-1β protein and pyroptotic cell death, next we determined whether caspase-1 is associated with TG-induced macrophage cell death. We found an increase in caspase-1 activity in TG-treated THP-1 macrophages and inhibition of caspase-1 activity using a specific inhibitor partially rescued cell death. These results suggest activation of the pyroptotic pathway by TG. This is the first report implicating the activation of caspase-1 and the triggering of the pyroptosis pathway in TG-induced macrophage cell death.

Key words triglyceride; macrophage; cell death; caspase-1; interleukin-1β

Atherosclerosis is a complex inflammatory vascular disease1–3 that leads to sudden cardiac death and acute myocardial infarction.4 The accumulation of oxidized low density lipoprotein (oxLDL) is considered crucial for developing atherosclerosis.1,5 Recently, hypertriglyceridemia has been proposed as a risk factor for atherosclerosis.6–9 Macrophages phagocytose excess lipids such as triglyceride (TG), which accumulate inside macrophages and result in the formation of foam cells. These foam cells subsequently die and promote further inflammation, plaque development, and rupture in atherosclerosis.10–15 Based on these observations, studies have focused on understanding the molecular mechanism of TG on macrophages, especially in cell death. TG has been reported to promote necrotic macrophage cell death by inducing mitochondria-mediated oxidative stress.11 However, the exact molecular mechanism by which TG accumulation induces macrophage cell death is not fully understood.

Cell death can occur through three different pathways—apoptosis, pyroptosis, and necrosis.16 Each pathway exhibits several overlapping features, but can also be distinguished by the presence of specific cellular and molecular changes. Pyroptosis is programmed cell death that results in cell lysis and causes inflammation of nearby tissues. Thus far, pyroptosis has only been described in macrophages and dendritic cells.18 Pyroptosis is characterized by activation of the cysteine protease caspase-1.19 Active caspase-1 has been detected in vulnerable plaques and ruptured lesions in patients dying of acute coronary events,17,18 implicating pyroptosis activation in this disease. In this study, we show that caspase-1 is an important factor in the TG-induced death of human THP-1-derived macrophages. To our knowledge, this is the first report implicating caspase-1 in TG-induced macrophage cell death. The identification of this molecular pathway may provide targets for therapeutic intervention.

MATERIALS AND METHODS

Materials TG emulsion (Lipofundin® MCT/LCT 20%) was purchased from B. Braun Melsungen AG (Melsungen, Germany). Lipofundin® MCT/LCT 20% was used to deliver TG into cells in previous studies.11,12 The composition of Lipofundin® MCT/LCT 20% is as follows: medium chain TG (100 g/L), soybean oil, glycerol, egg lecithin, all-rac-α-tocopherol, sodium oleate and water. Hereafter, Lipofundin® MCT/LCT 20% will be referred to as TG for convenience. Trypan blue stain solution and TRIZol® for RNA isolation were provided by Invitrogen (Carlsbad, CA, U.S.A.). Recombinant human interleukin (IL)-1β (rIL-1β) was obtained from PEPRO TECH (Rocky Hill, NJ, U.S.A.). The p38 mitogen activated protein kinase (MAPK) inhibitor (SB203580) was purchased from Calbiochem (Darmstadt, Germany). Caspase-1 substrate Ac-YVAD-p-nitroanilide was purchased from Bioanal (Plymouth Meeting, PA, U.S.A.). Caspase-1 specific inhibitor Z-YVAD-FMK was purchased from BioVision (Mountain View, CA, U.S.A.). Phorbol 12-myristate 13-acetate (PMA) and Oil red-O stain powder was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). 4′-6-Diamidino-2-phenylindole (DAPI) was purchased from Vector Lab (Burlingame, CA, U.S.A.).

Cell Culture and Differentiation Human acute monocytic leukemia cells (THP-1) were grown in RPMI media containing 10% fetal bovine serum (FBS) and penicillin-streptomycin. They were maintained at 37°C in a humidified atmosphere with 5% CO2. Cells were seeded in 6-well plates (1×106 cells/well) and treated with PMA (100 nm) for 48 h to differentiate into macrophages. Cells were then incubated with TG for the indicated times.

Oil Red-O Staining and Assessment Differentiated THP-1 cells (1×106 cells per well) were incubated with or
without TG for 24 h. Cell monolayers were washed twice with phosphate buffered saline (PBS) and fixed in 10% formaldehyde for 5 min. Oil red-O solution (0.2% (w/v) Oil red-O powder, 60% isopropanol, 40% water) was applied 1 mL per well for 30 min at room temperature. Cell monolayers were washed with 60% isopropanol for 5 s and washed twice with PBS. Cells were examined under a microscope. For quantification, 1 mL 100% isopropanol was added to the stained cell monolayers. After 5 min, the absorbance of the extract was assayed by spectrophotometer at 510 nm.

**DAPI Staining** THP-1 cells (2×10^5 cell per well) were seeded onto cover glass slides (24 mm×24 mm) inside 6-well culture plates and cultured with PMA (100 nm) for 48 h. The cells were then incubated with or without TG for 24 h. The cover glass containing cell monolayers was washed twice with PBS and fixed in 2% (w/v) paraformaldehyde for 5 min. After fixation, the cover glass was washed twice with PBS and incubated with DAPI stain solution in the dark for 10 min. The cover glass was placed on a microscope slide and observed under a fluorescence microscope (Olympus, Tokyo, Japan) at 430 nm.

**Trypan Blue Dye Exclusion Assay** Method details were previously described. In brief, cells were trypsinized and stained with trypan blue dye solution. Viable cells (i.e., trypan blue dye negative) were counted on a hemocytometer (Marienfeld, Lauda-Königshofen, Germany).

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)** Total RNA was extracted using Trizol® reagent according to the manufacturer’s instructions. cDNA was synthesized by reverse transcription with 2 μg of total RNA, 0.25 μg of random hexamer, and 200 U of MMLV-RT for 50 min at 37°C and for 15 min at 70°C. Subsequent PCR amplification using 0.2 U of Taq polymerase (Cosmogenetech, Seoul, Korea) was performed in a thermocycler using specific primers. Primer sequences are as follows: IL-1β; 5′-AGG CAT GGC AGA AGT ACC T-3′ (forward), 5′-CAG CTC TCT TTA GGA AGA C-3′ (reverse), IL-1R1; 5′-ATG AGA CAA TGG AAG TAG AC-3′ (forward), 5′-TAG ATG AAA ACA GAA CAC AC-3′ (reverse), tumor necrosis factor (TNF)-α; 5′-AGCCCA TGT TGT AGC AAA CC-3′ (forward), 5′-CTG AGT CGG TCA CCT TCT-3′ (reverse), IL-1β; 5′-TGG CCTGCT GTC AACCAG TAG AA-3′ (forward), 5′-GCC GAT TTC TTG GTC TCA AT-3′ (reverse), macrophage inflammatory protein (MIP)-1α; 5′-GCT GACT ATT TTG AGACG AGC AGC-3′ (forward), 5′-CCA GTC CATAGA GGT AGC-3′ (reverse), glyceraldehyde-3-phosphate dehydrogenase (GAPDH); 5′-GGG GAA GCT TGG TATCAA TGG-3′ (forward), 5′-GGT AGT GAT GGC ATG GACTG-3′ (reverse). GAPDH was used as an internal control. PCR products were electrophoresed on 1.5% (w/v) agarose gels containing 0.4% μg/mL ethidium bromide and gel images were taken using a Gel Doc™ XR+ system. Densitometric analysis was performed by Image Lab™ software (Bio-Rad, Hercules, CA, U.S.A.).

**Measurement of Caspase-1 Activity** Caspase-1 activity was measured as previously described. In brief, cells were lysed with a PBS buffer containing 1% Triton X-100 and protease inhibitor cocktail. They were then centrifuged at 19000×g for 10 min at 4°C. The supernatant was collected and the total protein concentration was quantified using Lowry protein assay (Bio-Rad, Hercules, CA, U.S.A.). Protein samples (90 μg) were mixed with Ac-YVAD-p-nitroanilide (200 μM) and incubated for 3 h at 37°C. Caspase-1 activity was determined by measuring absorbance at 405 nm.

**IL-1β Enzyme-Linked Immunosorbent Assay (ELISA)** Assay of IL-1β at 24 h: Differentiated THP-1 cells (1×10^6 cells per well) were incubated with or without TG for 24 h. The cell culture media was analyzed and the IL-1β concentration represented as pg/10^6 viable cells at 24 h.

Assessment of IL-1β at 6 h after media change: To assess the concentration of secreted IL-1β after 24 h post-TG culture, differentiated THP-1 cells (1×10^6 cells per well) were incubated with or without TG for 24 h. The cell culture media were aspirated and the cells were washed with PBS and cultured in serum-free medium another 6 h at 37°C. The cell culture media was analyzed and the IL-1β concentration represented as pg/10^6 viable cells at 6 h. IL-1β in culture supernatants were determined by Quantikine ELISA Human IL-1β/IL-1F2 kit (R&D Systems Inc., Minneapolis, MN, U.S.A.).

**Statistical Analysis** *p* values were calculated using either the Student’s *t*-test or two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test for multiple comparisons. Values are shown as mean and standard error of the mean (S.E.M.). Data were collected from three independent experiments.

**RESULTS**

**Accumulation of TG Induces Macrophage Cell Death** We examined whether TG accumulated inside THP-1 macrophages when TG was added to the cell culture media. PMA-differentiated THP-1 cells were incubated with TG (1 mg/mL) for 24 h and stained with Oil red-O. Prominent red droplets were found in the cytoplasm of TG-treated macrophages and negligible amounts of red droplets were found in macrophages without TG (Fig. 1A). We quantified the amount of intracellular TG by measuring the absorbance of Oil red-O stain after lipid extraction from TG treated cells. We found increased concentrations of intracellular TG proportionate to the amount of TG added to the culture (Fig. 1B). These results demonstrate that TG accumulates inside macrophages in a dose-dependent manner.

TG accumulation in the murine macrophage cell line J774.2 induces cell death. We previously showed that THP-1 macrophages treated with TG (1 mg/mL) for 24 h exhibited reduced viability. To expand upon this observation, differentiated THP-1 cells were treated with TG in a dose- and time-dependent manner. Viable cells were enumerated using trypan blue dye. THP-1 macrophages treated with TG showed a ca. 35% decrease in viability (*p*<0.001) when treated with 0.5 mg/mL of TG (Fig. 1C). In a time course experiment, TG (1 mg/mL) induced a ca. 30% decrease in cell viability (*p*<0.001) beginning at 24 h of TG treatment (Fig. 1D). These results demonstrate that TG accumulation in THP-1 macrophages induces cell death in a dose- and time-dependent manner. To determine the pathway through which cell death is induced, THP-1 macrophages were treated with TG (1 mg/mL) for 24 h and the cells stained with DAPI. We found that TG treated cells showed condensed nuclei characteristic of cells undergoing apoptosis or pyroptosis (Fig. 1E). Taken together, these results suggest that uptake of TG induced apoptotic or pyroptotic cell death in THP-1 macrophages.

**TG Induces Decreased Expression of IL-1β mRNA but...**
Increased Secretion of IL-1β Protein The inflammatory cytokines TNF-α, IL-18, and IL-1β are involved in atherosclerosis development. In addition, MIP-1α is secreted by activated macrophages in response to various stimulants. To determine if TG treatment affects the expression of these cytokines, THP-1 macrophages were treated with TG and cytokine expression was examined by PCR analysis. The expression of IL-1β decreased markedly in response to TG treatment, as previously reported. However, the expression of the IL-1R1, TNF-α, IL-18, and MIP-1α remained unchanged (Fig. 2A). To determine if the decrease in IL-1β mRNA levels correlated with decreased IL-1β protein levels, we examined the levels of secreted IL-1β in THP-1 macrophages treated with TG. Since TG can induce cell death in a subset of cells, we normalized the IL-1β protein levels to viable cell number. TG treatment for 24 h resulted in dose-dependent increase in secreted IL-1β protein (Fig. 2B, upper panel). To more directly assess secreted IL-1β by viable cells, the cell supernatant was...
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replaced with fresh serum-free media for 6 h and IL-1β ELISA performed (Fig. 2B, lower panel). Contrary to expectations, both results show a dose-dependent increase in IL-1β secretion per cell after TG treatment suggesting that although TG induces a decrease in IL-1β mRNA the secretion of IL-1β protein by viable cells is increased (Fig. 2B).

Previously, we reported that activation of p38 MAPK is involved in the mechanism of decreased IL-1β expression by TG treatment. We determined if p38 MAPK is involved in TG-induced cell death using a p38 MAPK inhibitor (SB203580). Our results indicate that the addition of the p38 MAPK inhibitor does not prevent TG-mediated cell death suggesting that the p38 MAPK which is involved in down-regulation of IL-1β mRNA expression is not involved in cell viability (Fig. 2C). Next, we determined whether the increase in secreted IL-1β was directly responsible for TG-induced cell death. THP-1 macrophages were treated with TG (1 mg/mL) and different concentrations of rIL-1β (0, 5, 10, 15, 20 nM) for 24 h. The number of viable cells in THP-1 macrophages without TG and rIL-1β treatment was set as 100%. Data from three independent experiments. Values are shown as mean and S.E.M.; p-values were determined by Student’s t-test.

Fig. 2. TG-Induced THP-1 Macrophages Have Decreased IL-1β mRNA Expression but Increased IL-1β Protein Secretion

(A) Cells were treated with TG (0, 0.1, 0.5 mg/mL) for 24 h. cDNA was subjected to PCR to amplify TNF-α, IL-18, MIP-1α, IL-1β and IL-1β receptor. PCR products were resolved on a 1.5% agarose gel. GAPDH was used as an internal control. (B) IL-1β ELISA. Cells were cultured with TG (1 mg/mL) for 24 h and the IL-1β levels in the cell culture supernatant were measured and represented as pg/10^6 viable cells (upper panel). After removing the supernatant of TG-treated cell for 24 h, serum-free culture media was added and incubated, then the supernatant harvested after 6 h for analysis (lower panel). (C) Cells were treated with TG (1 mg/mL) for 24 h with or without the p38 MAPK inhibitor (SB203580) and viable cells enumerated using trypan blue. Data from three independent experiments. (D) Cells were treated with TG (1 mg/mL) and rIL-1β for 24 h. The number of viable cells in THP-1 macrophages without TG and rIL-1β treatment was set as 100%. Data from three independent experiments. Values are shown as mean and S.E.M.; p-values were determined by Student’s t-test.
induces a decrease in IL-1β mRNA but an increase in IL-1β protein. However, these changes are not the direct cause of TG-induced macrophage cell death.

**TG Induces Cell Death via Uprgulation of Caspase-1 Activity** The IL-1β ELISA results suggest a post-translational regulation of IL-1β; that is, cleavage of latent IL-1β to active form of IL-1β. IL-1β is processed by caspase-1, which is known to induce cell death via pyroptosis in response to a variety of stimuli. Therefore, we determined whether caspase-1 is involved in TG-induced macrophage cell death. Surprisingly, caspase-1 activity was found to increase in THP-1 macrophages in a TG dose-dependent (Fig. 3A) and time-dependent manner (Fig. 3B). To elucidate whether increased caspase-1 activity is responsible for cell death, THP-1 macrophages were treated with TG in the absence or presence of caspase-1-specific inhibitor (Z-YVAD-FMK) and viable cells were enumerated. Z-YVAD-FMK was found to partially inhibit cell death in an inhibitor dose-dependent manner (Fig. 3C). However, IL-1β mRNA expression remained unchanged in the presence of the caspase-1 inhibitor (Z-YVAD-FMK) suggesting additionally that change in mRNA expression is not directly responsible for TG induced macrophage death (Fig. 3D). We conclude that TG-induced macrophage cell death is mediated in part by the increase in caspase-1 activity.

**DISCUSSION**

An increased blood TG level is a known risk factor for atherosclerosis. One proposed reason for this is that TG induces macrophage cell death which subsequently leads to increased inflammation, thereby exacerbating the formation of atherosclerotic lesions. However, the exact mechanism by which TG induces macrophage cell death is unknown. In the present study, we found that TG stimulates caspase-1 activity in macrophages, which contributes to macrophage cell death. Many inflammatory cytokines are associated with cell death, and a subset of these cytokines is known to trigger macrophage death. We examined the expression of three cytokines (TNF-α, IL-18, and IL-1β) that are reported to promote cell death during the development of atherosclerosis. We found that IL-1β mRNA expression was decreased but the IL-1β protein levels increased with TG treatment (Figs. 2A, B). Inhibition of the p38 MAPK which is involved in the mechanism of decreased IL-1β expression by TG treatment did not prevent TG-mediated cell death (Fig. 2C). Exogenous supplementation of rIL-1β did not influence TG-induced macrophage cell death (Fig. 2D). These results suggest that decreased IL-1β mRNA expression and increased IL-1β protein secretion are not directly responsible for TG induced macrophage death. Further studies are needed to elucidate the discrepancy between IL-1β mRNA expression and IL-1β protein secretion after TG treatment.

Caspase-1 induces cell death via the pyroptotic pathway. Our data suggests that caspase-1 activity increases in macrophages in response to TG treatment (Fig. 3) and characteristics of cells undergoing pyroptosis are observed (Fig. 1E), implying that the pyroptotic pathway is one possible mechanism by which TG induces macrophage cell death. However, the inhibition of caspase-1 using a specific inhibitor did not completely prevent cell death, suggesting involvement of a caspase-1-independent pathway. Recently, it has been reported that TG-triggered macrophage cell death is also mediated via a caspase-3/7-dependent apoptotic pathway. It was also reported that caspase-1 is involved in the mitochondrial apoptotic pathway due to activation of caspase-9 and caspase-3. Taken together with our results, we suggest...
that TG-induced macrophage death is mediated by activation of both the caspase-1-dependent pyroptotic pathway and the caspase-3/7-dependent apoptotic pathway.

In conclusion, we report for the first time that caspase-1 plays a critical role in TG-induced macrophage cell death. This suggests that TG-induced cell death occurs in part through activation of the pyroptotic pathway. Although further studies are needed to identify signaling molecules involved in caspase-1 activation, the identification of this pathway will likely provide clues in understanding how TG contributes to the development of atherosclerosis.

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