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ORIGINAL ARTICLE

Cathepsin B Is Implicated in Triglyceride (TG)-Induced Cell Death of Macrophage

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중성지방에 의한 대식세포 사멸 과정에서 Cathepsin B의 영향

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ABSTRACT

Macrophage cell death contributes to the formation of plaque, leading to the development of atherosclerosis. The accumulation of triglyceride (TG) is also associated with the pathogenesis of atherosclerosis. A previous study reported that TG induces the cell death of macrophages. This study examined whether the cytoplasmic release of cathepsin B from lysosome is associated with the TG-induced cell death of macrophage. The release of cathepsin B was increased in the TG-treated THP-1 macrophages, but the TG treatment did not affect cathepsin B expression. Furthermore, the inhibition of cathepsin B by its inhibitor, CA-074 Me, partially inhibited the TG-induced cell death of macrophage. TG-triggered macrophage cell death is mediated by the activation of caspase-1, -2, and apoptotic caspases. Therefore, this study investigated whether cathepsin B is implicated in the activation of these caspases. The inhibition of cathepsin B blocked the activation of caspase-7, -8, and -1 but did not affect the activity of caspase-3, -9, and -2. Overall, these results suggest that TG-induced cytoplasmic cathepsin B causes THP-1 macrophage cell death by activating caspase-1, leading to subsequent activation of the extrinsic apoptotic pathway.

INTRODUCTION

Atherosclerosis is a prevalent disease caused by clogged arteries with lipid-rich plaque, which, in turn, progress myocardial ischemia [1]. The prevalence of clinical atherosclerosis per 1,000 individuals was 101.11 in 2015 in South Korea [2]. While a variety of cell types contribute to the pathogenesis of atherosclerosis, macrophages play a pivotal role in terms of plaque formation and development [3]. In the initial stage of atherosclerosis, macrophages engulf cholesterol-rich lipoprotein via specific receptors and subsequently are converted to foam cells, which is a crucial step for the development of plaque formation [4]. As plaques develop, macrophage cell death increases, which contributes to the development of vulnerable plaques [5]. Previously, our group have reported that TG induces cell death of macrophages implying the implication of TG in the

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pathogenesis of atherosclerosis [6, 7].

Cathepsins are cysteine proteases which are localized in the early endosome and lysosomes of immune cells such as macrophages [8]. Among 11 human cathepsins, cathepsin B is the most well-studied protease regarding cell death. The released cathepsin B in the cytosol cleaves proapoptotic molecule, BID, which in turn leads to the release of cytochrome c from mitochondria and subsequently induces apoptosis in a variety of cell types [9]. Interestingly, multiple investigations have supported that cathepsin B is associated with the pathogenesis of atherosclerosis. For example, upregulation of cathepsin B was reported in atherosclerosis animal model, apoE$^{-/-}$ mice [10]. In addition, cathepsin B can degrade the extracellular matrix in the arterial intima, which promotes inflammatory response as well as the development of vulnerable plaques [11].

In the present study, we aimed to understand the role of cathepsin B in TG-induced macrophage cell death using differentiated human THP-1 macrophages. We found that challenge of TG induced release of cathepsin B from the lysosome to the cytoplasm, which subsequently activates extrinsic apoptotic pathways. Moreover, we showed cathepsin B is a prerequisite for caspase-1 activation, which is the same observance with the very recent report [12]. Our results show that TG-induced macrophage cell death is mediated by the cytosolic release of cathepsin B from the lysosome.

MATERIALS AND METHODS

1. Materials

Lipofundin® MCT/LCT 20% was obtained from B. Braun Melsungen AG (Melsungen, Germany). Lipofundin® MCT/LCT 20% was used to deliver TG into cells as described in previous study [13]. Hereafter, Lipofundin® MCT/LCT 20% will be referred to as TG for convenience. The caspase-1 substrate, Ac-YVAD-p-nitroanilide (Ac-YVAD-pNA) was purchased from Biomal (Plymouth Meeting, PA, USA). The caspase-2 substrate, Ac-VAVAD-pNA was obtained from Sigma-Aldrich (St. Louis, MO, USA). CA-074 Me (cathepsin B specific inhibitor) was purchased from Calbiochem (Darmstadt, Germany). Antibodies specific for caspase-3, -7, -8 and -9, as well as PARP, were purchased from Cell Signaling technology (Danvers, MA, USA). Antibody specific for cathepsin B was obtained from Santa Cruz biotechnology (Santa Cruz, CA, USA).

2. Cell culture

The THP-1 human acute monocytic leukemia cell line (ATCC, Manassas, VA, USA) was cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin and maintained at 37°C in a humidified atmosphere with 5% CO_2. To induce differentiation of THP-1 monocytic cells into macrophages, cells were plated in 6-well plates at a density of 1×10^6 cells/well and incubated with 200 nM phorbol 12-myristate 13-acetate (PMA) for 48 h [14, 15].

3. Oil red-O staining and assessment

THP-1 cells were seeded on cover-glass (24×24 mm) and incubated with 200 nM PMA for differentiation into macrophages. Then, the cells were incubated with or without TG for 24 h. Cell monolayers were washed twice with Dulbecco's phosphate buffered saline (DPBS) and fixed in 10% formaldehyde for 5 min. 1 mL of 0.2% Oil red-O solution (Sigma-Aldrich) was added to well and incubated for 30 min at room temperature. Cell monolayers were washed with 60% isopropanol for 5 sec and washed twice with DPBS. Cells were examined under a microscope. For quantification, 1 mL of 100% isopropanol was added to the stained cell monolayers. After 5 min, the absorbance of the extract was assayed by UV/Vis spectrophotometer at 510 nm (Beckman Coulter, Fullerton, CA, USA).

4. Trypan blue dye exclusion assay

Method were previously described in detail [7]. In brief, THP-1 cells were trypsinized and 10 μL of 0.4% trypan blue stain solution was mixed with 10 μL of the trypsinized cells at a 1:1 ratio. Viable cells (unstained

cells) were counted on a hemocytometer (Marienfeld, Lauda-Königshofen, Germany).

5. Subcellular fractionation

Method were previously described in detail [16]. The THP-1 cells were plated at a density of 6×10^6 cells on each culture plate (100 mm×20 mm) and incubated with 200 nM PMA for 48 h. Thereafter, differentiated THP-1 macrophage were incubated with TG for an additional 24 h. Scraped THP-1 cells were washed with cold PBS and cells were pelleted by centrifugation at 1,000 g for 5 min. All samples were resuspended in 350 μL of buffer containing 250 mM sucrose, 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 1% (v/v) protease inhibitor cocktail and 1% (v/v) phosphatase inhibitor cocktail and incubated on ice for 30 min. Then, samples were vortexed at maximum speed for 15 sec and centrifuged at 7,500 g for 15 min. The supernatant was used as cytosolic fractions.

6. Western-blot analysis

THP-1 cells were washed with PBS and lysed at 4˚C with lysis buffer including 1% Triton X-100, protease inhibitor cocktail (Sigma-Aldrich), phosphatase inhibitor cocktail (Roche, Mannheim, Germany), and PBS. Cell lysates were clarified and the supernatants subjected to Western blotting as described previously [17].

7. RNA extraction and semi-quantitative reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from THP-1 cells using Trizol® reagent according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcriptase PCR (RT-PCR) was performed as previously described [6]. Complementary DNA (cDNA) was synthesized by reverse transcription with 2 μg of total RNA, 0.25 μg of random hexamer (Invitrogen) and 200 U of MMLV-RT (Invirogen) for 10 min at 25˚C, 50 min at 37˚C and 15 min at 70˚C. cDNA was PCR amplified using Prime Taq premix PCR kit (Genet Bio, Chungnam, Korea) for 25~40 cycles using specific primers. Primer sequences are as follows: Cathepsin B; 5'-ACA GTG TCC CAC CAT CAA AG-3' (forward), 5'-CAC CAT TAC AGC CGT CCC-3' (reverse) and GAPDH; 5'-CGG GAA GCT TGT CAT CAA TGG-3' (forward), 5'-GGC AGT GAT GGC ATG GAC TG-3' (reverse). GAPDH was used as an internal control. PCR amplicons were electrophoresed on 2% (w/v) agarose gels including 0.5 μg/mL ethidium bromide and the amplicon size determined by comparison to 100 bp DNA ladder marker (Intron, Gyeonggi, Korea). Gel images were obtained using Gel Doc™ XR+ system (Bio-Rad, Hercules, CA, USA). The PCR amplicon band intensity was measured and normalized against GAPDH using Image Lab™ software (version 4.1, Bio-Rad).

8. Measurement of caspase-1 and caspase-2 activities

The activity of caspase-1 and caspase-2 was measured as previously described [18]. Briefly, THP-1 cells were lysed with PBS buffer containing 1% Triton X-100 and then centrifuged at 19,000 g for 10 min at 4˚C. The supernatant was collected and the total protein concentration was quantified. To measure caspase-1 activity, 90 μg of protein sample was mixed with 200 μM of the caspase-1 substrate Ac-YVAD-pNA in 150 μL of PBS. To measure caspase-2 activity, protein was mixed with the caspase-2 substrate Ac-VAVAD-pNA in PBS. Mixtures were incubated for 3 h at 37˚C, and the activity was determined by measuring the absorbance at 405 nm.

9. Statistical analysis

Statistical analysis was performed with student's t-test using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Values are expressed as the mean and standard error of the mean (SEM). Each experiment was conducted three times and the date were pooled for analysis. Differences were considered to be statistically significant at $^*P<0.05$, $^{**}P<0.01$, or $^{***}P<0.001$.

RESULTS

1. TG accumulation induces cytoplasmic release of cathepsin B from lysosome

We previously reported that TG-induced macrophage cell death is caused by caspase-1 [6]. Several studies showed that the activation of caspase-1 can also occur via lysosomal rupture and subsequent cytosolic release of cathepsin B [19, 20]. Therefore, we first investigated whether the release of cathepsin B is involved in TG-induced THP-1 macrophage cell death. We first reconfirmed whether TG accumulates in THP-1 macrophages when it is added in a cell culture media. PMA-differentiated THP-1 macrophages were incubated with TG for 24 h and then stained with Oil-red-O solution. Unlike in macrophages without TG treatment, stained droplets were more prominently found in the cytoplasm of TG-treated macrophages (Figure 1). Next, to elucidate whether the cytoplasmic protein level of cathepsin is influenced by TG treatment, the cytoplasmic fraction which do not include lysosomes was obtained through the process of subcellular fraction. TG treatment increased the cytoplasmic protein level of cathepsin B in a dose- and time-dependent manner, but the total protein level of cathepsin B in THP-1 macrophages was unchanged in response to TG (Figure

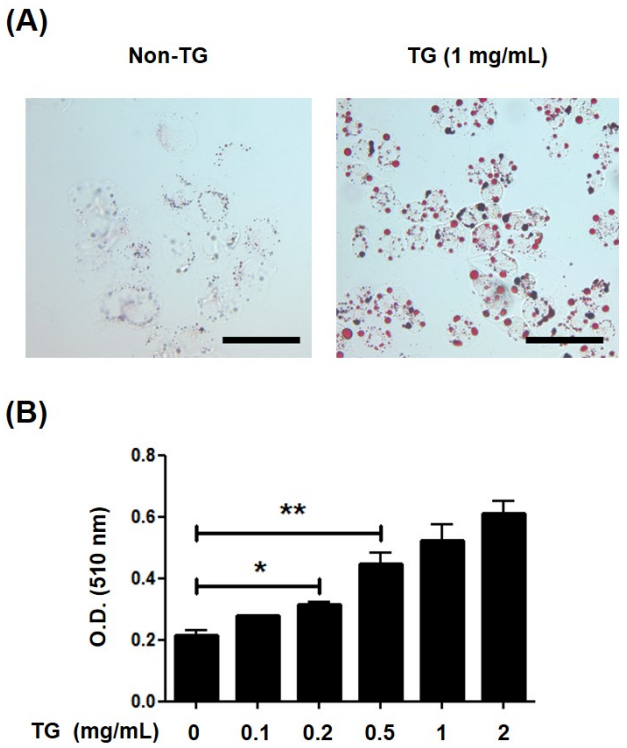

Figure 1. The accumulation of TG in THP-1 macrophages forms foam cells. (A) THP-1 cells were differentiated with 200 nM PMA for 48 h and incubated with or without 1 mg/mL of TG for additional 24 h. Oil red-O staining was performed to detect lipid droplets in cells (scale bars, 100 μm). (B) Quantitative analysis of Oil red-O uptake was performed after cells were cultured with TG and then stained with Oil red-O. The dye was extracted and the absorbance was measured by spectrophotometry at 510 nm. All data are expressed as the mean±SEM of three independent experiments. P-values were determined with Student's t-test. *P<0.05, **P<0.01.

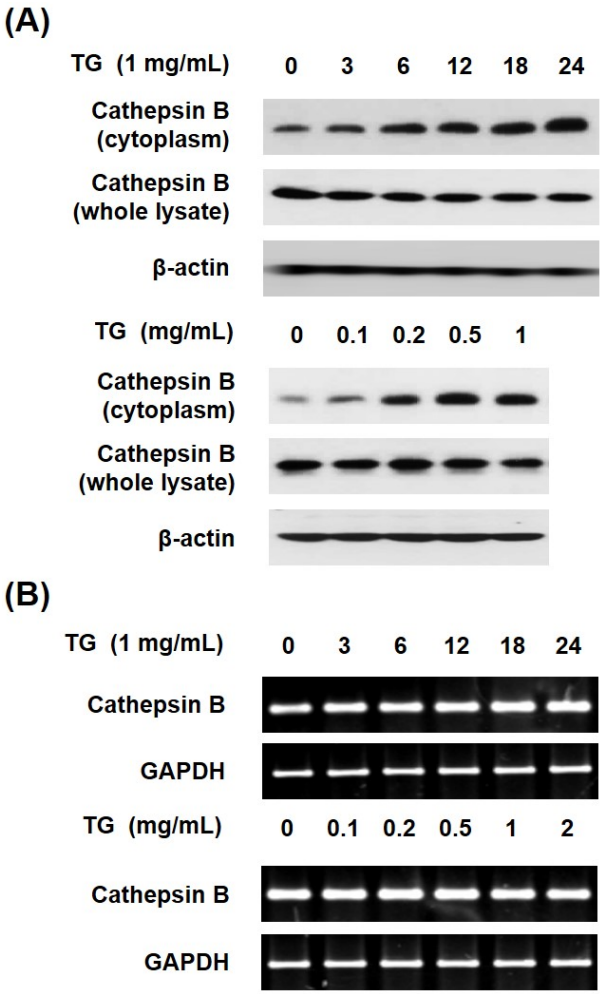

Figure 2. TG induces cytoplasmic release of cathepsin B. (A) THP-1 macrophages were incubated with the indicated concentration of TG for the indicated times, and the cytosol and residues were separated using gradient centrifuges. The cathepsin B was detected by Western blotting. (B) THP-1 macrophages were incubated with the indicated concentration of TG for the indicated times, and expression of cathepsin B mRNA was assessed by RT-PCR.

2A). Furthermore, when the RNA level of cathepsin B was examined by RT-PCR analysis, the expression level of cathepsin B was unchanged in the presence of TG in THP-1 macrophages (Figure 2B). These results imply that the increase of cathepsin B in the cytoplasm of TG-treated THP-1 macrophages is caused by TG-triggered cytosolic release of cathepsin B from lysosome and not by the increased expression of cathepsin B.

2. TG-stimulated cytoplasmic release of cathepsin B involves in THP-1 macrophage cell death

Next, to elucidate the role of cathepsin B in TG-induced macrophage cell death, it was examined whether cell viability was influenced by treatment with a cathepsin B inhibitor, CA-074 Me. THP-1 macrophages were differentiated with PMA and incubated with TG in the absence or presence of CA-074 Me for 24 h and viable cells were enumerated. We found that it partially recovered cell viability of TG-treated THP-1 macrophages in an inhibitor dose-dependent manner (Figure 3). This result suggests that the cytosolic release of cathepsin B by the accumulation of TG leads to THP-1 macrophage cell death.

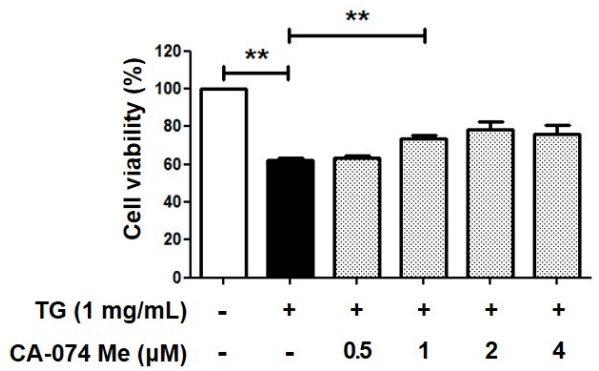

Figure 3. TG-stimulated increase of cytoplasmic cathepsin B is associated with TG-triggered macrophage cell death. THP-1 macrophages were incubated with 1 mg/mL of TG in the presence of cathepsin B inhibitor, CA-074 Me for 24 h. Then, trypan blue exclusion assay was performed to enumerate viable cells. The number of viable cells in THP-1 macrophages without TG treatment was set as 100%. All data are expressed as the mean±SEM of three independent experiments. *P*-values were determined with Student's t-test. $^{**}P<0.01$.

3. TG-induced cytoplasmic cathepsin B release leads to THP-1 macrophage cell death via the extrinsic apoptotic pathway

TG induced-macrophage cell death is reportedly mediated by classical apoptotic caspases [7]. Therefore, we examined the association of cathepsin B with the activation of caspases in TG-induced THP-1 cell death. When THP-1 macrophages were incubated with TG and cathepsin B inhibitor, CA-074 Me for 24 h, the cleavage of PARP, which was increased by TG treatment, was partially reduced (Figure 4A). In addition, CA-074 Me blocked the cleavage of caspase-7, which is known to cleave PARP, however, caspase-3 was not affected

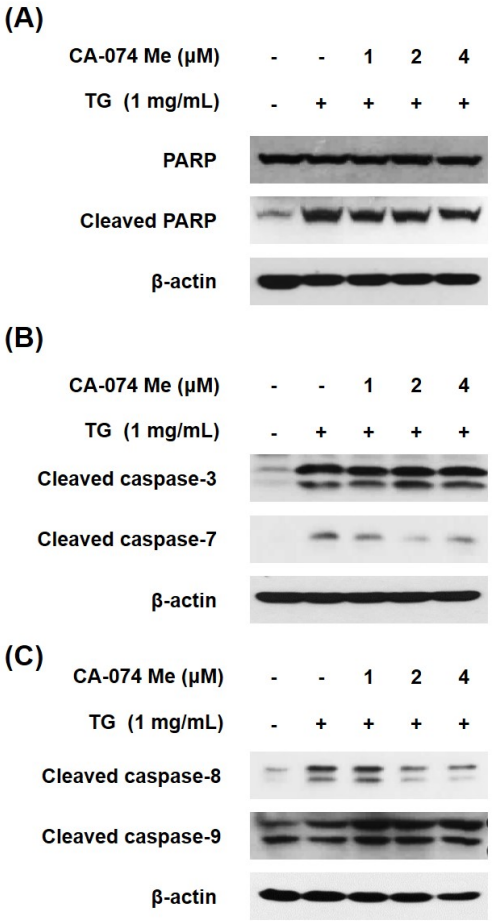

Figure 4. TG-induced cytoplasmic cathepsin B affects THP-1 macrophage cell death via the extrinsic apoptotic pathway. THP-1 macrophages were incubated with of TG in the presence of cathepsin B inhibitor, CA-074 Me for 24 h. (A) The cleaved form of PARP, (B) cleavage of caspase-3 and -7, and (C) cleavage of caspase-8 and caspase-9 were detected by Western blotting.

(Figure 4B). Caspase-8 and -9 are upstream molecules of caspase-7, and therefore, we examined their activity in the presence or absence of CA-074 Me. The cleavage of caspase-8 was decreased by CA-074 Me in a dose-dependent manner, whereas caspase-9 showed no significant changes in response to CA-074 Me in TG-treated macrophages (Figure 4C). These results suggest that TG-induced cytoplasmic cathepsin-B release leads to THP-1 macrophage cell death via the extrinsic apoptotic pathway.

4. Cathepsin B is associated with activation of caspase-1 in TG-triggered macrophage cell death

Previous studies have reported that caspase-1 and -2 is implicated in TG-induced THP-1 macrophage cell death [6, 7]. To determine whether the increase of cytoplasmic cathepsin B is involved in the activation of caspase-1 and -2 in TG-treated THP-1 macrophages, PMA-differentiated THP-1 macrophages were incubated with TG in the presence or absence of CA-074 Me for 24 h, and the activation of the caspase-1 and -2 was analyzed. Ca-074 Me reduced the activity of caspase-1, which was increased by TG treatment (Figure 5A). However, the inhibition of cathepsin B did not affect the TG-induced activation of caspase-2 in THP-1 macrophages (Figure 5B). These data suggest that released cytosolic cathepsin B is an upstream molecule of caspase-1 in TG-treated THP-1 macrophages.

DISCUSSION

Multiple lines of evidence support that raised TG concentration in blood can be accumulated in the arterial wall and cause atherosclerosis [21]. In a similar context, the role of cathepsin B in the development of atherosclerosis is well documented, such as involvement in apoptosis of macrophage and degradation in fibrous cap of atherosclerotic plaque [22]. However, the link between TG and cathepsin B, which contributes to the pathogenesis of atherosclerosis, has not been studied. In this study, we have elucidated that TG-induced macrophage cell death is mediated cytosolic release of cathepsin B from lysosome. This is the first report to demonstrate that TG can liberate cathepsin B from lysosome to cytosol, followed by activation of extrinsic caspase cascade in the human macrophage cell line.

Macrophages are crucial cells contributing to the formation of plaques in atherosclerosis, and its apoptosis promotes the conversion from stable to unstable plaques [23]. It has been reported that cathepsin B is implicated in extracellular degradation, which is highly associated with the development of vulnerable plaque [24]. In addition, cathepsin B is highly expressed and appears in the cytoplasm of apoptotic macrophages

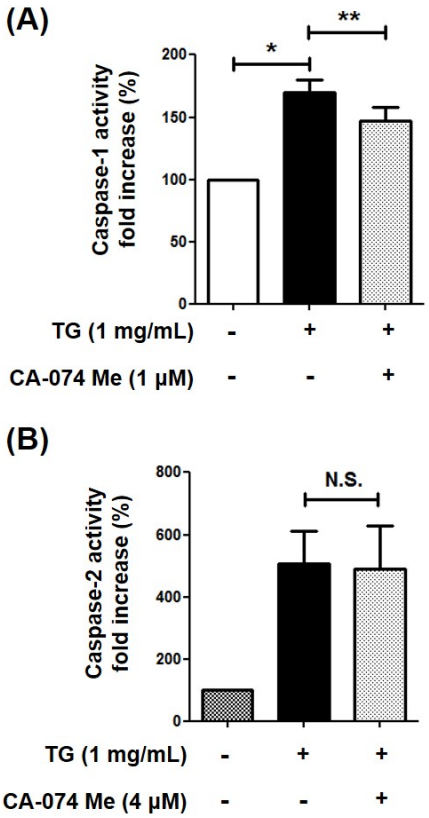

Figure 5. Cathepsin B is an upstream molecule of caspase-1 in TG-triggered macrophage cell death, but not caspase-2. (A) THP-1 macrophages were incubated with TG in the absence or presence of the CA-074 Me for 24 h, after which caspase-1 activity was assessed. Absorbance of THP-1 macrophages without treatment with TG was set as 100%. (B) THP-1 macrophages were incubated with TG in the absence or presence of the CA-074 Me for 24 h and caspase-2 activity was assessed. All data are expressed as the mean±SEM of three independent experiments. P-values were determined with Student's t-test. *$P<0.05$, ** $P<0.01$.

located in the atherosclerotic lesion [25]. So far, the well-known mechanism by which cathepsin B mediates macrophage cell death is highly dependent on the intrinsic caspase cascade, including Bid and caspase-9 [26]. However, Results in the present study showed that TG-mediated cytosolic release of cathepsin B leads to cell death of macrophages is an extrinsic caspase cascade instead of an intrinsic caspase cascade.

In this study, we observed that TG treatment induces the translocation of cathepsin B to cytosol in macrophage. Previously, we have shown that TG treatment results in increase of TNF-α in the THP-1 cells [27]. Since it has been reported that TNF-α triggers the cellular redistribution of the proteins including cathepsin B, it is probably that TG-induced cytosolic release of cathepsin B can be mediated via TG-stimulated increase of TNF-α [28]. In conclusion, we report for the first time that cathepsin B plays a role in TG-stimulated cell death of macrophages. This suggests that TG-induced macrophage death occurs in part via cytosolic release of cathepsin B from lysosome. Our results will likely provide clues in understanding the mechanisms by which TG implicates in the development of atherosclerosis.

요 약

대식세포사멸은 죽상판 형성에 영향을 미쳐 죽상동맥경화증 발병에 관여하는 것으로 알려져 있다. 중성지방 역시 죽상동맥경화 발병에 기여한다고 알려져 있는데 최근 본 연구팀에서는 중성지방이 대식세포사멸을 유발한다는 결과를 확인하였다. 본 연구에서는 cathepsin B가 중성지방에 의해 유발되는 대식세포사멸 과정에 관여하는지 확인하고자 연구를 진행하였다. THP-1 대식세포에 중성지방 처리 시 cathepsin B의 발현량에는 변화가 없고 리소좀에 있던 cathepsin B가 세포질로 방출되어 세포질의 cathepsin B가 증가한 것을 확인하였다. 다음으로 cathepsin B 억제제인 CA-074 Me를 처리 시 중성지방에 의해 유도되는 대식세포사멸이 일부 회복되는 것을 확인하였다. 본 연구팀의 이전 연구에서 중성지방에 의한 대식세포사멸이 caspase-1, -2 및 apoptotic caspase 활성화를 매개로 일어남을 확인하였기 때문에 본 연구에서는 이러한 caspase 활성경로와 cathepsin B와의 연관성에 대해 연구하였다. cathepsin B 억제시 caspase-7, -8 및 -1의 활성은 억제되었으나, caspase-3, -9 및 -2는 활성에는 영향을 미치지 않음을 알 수 있었다. 정리하면, 중성지방에 의해 세포질로 방출된 cathepsin B는 caspase-1 활성화에 기여하고, 활성화된 caspase-1은 외인성 apoptotic caspase 경로를 활성화하여 THP-1 대식세포사멸을 유발한다는 것을 알 수 있다.

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Conflict of interest: None

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