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Increased expression of interleukin-1β in triglyceride-induced macrophage cell death is mediated by p38 MAP kinase

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Triglycerides (TG) are implicated in the development of atherosclerosis through formation of foam cells and induction of macrophage cell death. In this study, we report that addition of exogenous TG induced cell death in phorbol 12-myristate 13-acetate-differentiated THP-1 human macrophages. TG treatment induced a dramatic decrease in interleukin-1β (IL-1β) mRNA expression in a dose- and time-dependent manner. The expression of granulocyte macrophage colony-stimulating factor and platelet endothelial cell adhesion molecule remained unchanged. To identify signaling pathways involved in TG-induced downregulation of IL-1β, we added p38 MAPK, protein kinase C (PKC) or c-Raf1 specific inhibitors. We found that inhibition of p38 MAPK alleviated the TG-induced downregulation of IL-1β, whereas inhibition of PKC and c-Raf1 had no effect. This is the first report showing decreased IL-1β expression during TG-induced cell death in a human macrophage line. Our results suggest that downregulation of IL-1β expression by TG-treated macrophages may play a role during atherogenesis. [BMB Reports 2012; 45(7): 414-418]

INTRODUCTION

Atherosclerosis is a complex vascular disease and causes cardiac cell death and myocardial infarction through endothelial activation, cellular influx, and production of mediators and cytokines (1-4). A characteristic feature of atherosclerosis is the migration of macrophages to the lesion to become foam cells, which in turn, accelerate the progression of atherosclerotic lesions (2, 3, 5, 6). The uptake of oxidized low density lipoprotein (oxLDL) and accumulation of triglyceride (TG) by macrophages are important contributing factors during the development of atherosclerosis (7). TG accumulation in macrophages causes oxidative stress and further contributes to the formation of the foam cell phenotype (8-10).

Death of foam cells in atherosclerotic lesions can be triggered under a variety of conditions such as hypoxia, ATP depletion, a high concentration of oxLDL, and intracellular accumulation of unesterified or free cholesterol (11, 12). Inadequate clearance of dying foam cells results in an increased inflammatory response, which further exacerbates disease progression (13-18). In addition to the role of TG in foam cell formation, Aronis *et al.* showed that TG stimulates cell death in a mouse macrophage line (J774.2) by increasing oxidative stress (9, 10). Furthermore, very low-density lipoprotein (VLDL), a triglyceride-rich lipoprotein, and its oxidized form, oxVLDL, induce endothelial dysfunction by modulating genes involved in cell activation, damage, and proliferation (19).

Proinflammatory cytokines mediate the development of atherosclerosis by promoting thrombus formation and facilitating the development of acute coronary syndrome (20, 21). In addition, proinflammatory cytokines destabilize atherosclerotic plaques by promoting cell death and matrix degradation (21). The potent proinflammatory cytokine interleukin-1β (IL-1β) can induce cell death in vascular smooth muscle cells (VSMCs) (22) and human pancreatic epithelial cells (23).

TG is a critical and a well-documented risk factor for atherogenesis. Furthermore, TG-induced macrophage death is implicated in the development of atherosclerosis. However, the exact mechanism by which TG promotes macrophage death is unclear (9, 10). In this report, we used a human THP-1-derived macrophage line and demonstrate that addition of exogenous TG to macrophages induced cell death. We further show that TG induced a dramatic decrease in IL-1β expression in macrophages in a p38 MAPK dependent manner.

RESULTS AND DISCUSSION

TG reduces viability of THP-1 macrophages

TG has a cytotoxic effect on the murine macrophage line J774.2 (9, 10). However, it has not been demonstrated whether TG exerts a cytotoxic effect on human macrophages. To determine whether TG induces death of human macrophages, we first treated human THP-1 cells with phorbol 12-myristate 13-acetate (PMA) (100 nM) for 48 hr to differentiate THP-1 cells into

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macrophages. THP-1-derived macrophages were then incubated in the presence or absence of TG (1 mg/ml) for 24 hr. We found that TG-treated macrophages showed reduced cell viability compared to that in untreated macrophages when observed under a microscope (Fig. 1A). We enumerated viable cells using the trypan blue dye exclusion assay and found that TG-treated macrophages exhibited a ~30% decrease in cell number compared to that in untreated macrophages ($P < 0.001$). This result was consistent with previous reports showing that TG treatment induces cell death in murine macrophages (9, 10).

TG downregulates IL-1β expression

TG stimulates macrophage death; thus, increasing the inflammatory response in atherosclerotic lesions resulting in aggravation of atherosclerosis (13-18). We examined the expression levels of genes involved in apoptosis, proliferation, and atherogenesis to elucidate the mechanism by which TG causes macrophage death. We treated THP-1-derived macrophages with different concentrations of TG for 24 hr and then examined mRNA expression levels by polymerase chain reaction (PCR) analysis. We first examined expression of two important genes in atherosclerotic lesion development such as granulocyte-macrophage colony-stimulating factor (GM-CSF), which promotes macrophage proliferation in atherosclerotic lesions (24), and platelet endothelial cell adhesion molecule (PECAM-1), which is important for leukocyte migration (25). Our results showed that TG treatment had no effect on either GM-CSF or PECAM-1 expression (Fig. 2A). We next examined whether TG treatment affected expression of the antiapoptotic molecule B-cell lymphoma 2 (Bcl-2) and the pro-apoptotic molecule Bcl-2 homologous antagonist/killer 1 (BAK-1), both of which play critical roles during apoptosis. We found that both Bcl-2 and BAK-1 showed a modest change in expression by PCR analysis; Bcl-2 gene expression decreased gradually, whereas pro-apoptotic BAK-1 gene expression increased gradually in a TG dose-dependent manner (Fig. 2A). Decreased Bcl-2 and increased BAK-1 expression was consistent with our current cell viability data demonstrating that addition of exogenous TG to THP-1 derived macrophages reduced their viability (Fig. 1). In support of our findings, previous reports have shown that TG stimulates the apoptotic pathway in macrophages (26) and that upregulation of Bcl-2 protects macrophages from apoptosis (27). Taken together, these data strongly suggest that the decrease in macrophage viability after TG treatment was due to activation of the apoptotic pathway.

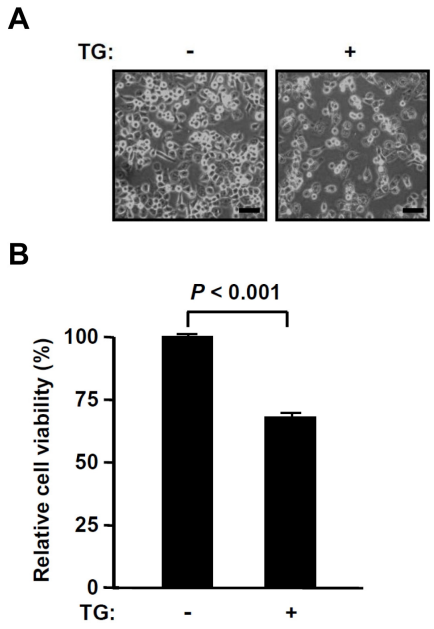

Fig. 1. Triglycerides (TG) reduce THP-1 macrophage viability. THP-1 cells were differentiated into macrophages by treatment with phorbol 12-myristate 13-acetate (PMA) (100 nM) for 48 hr. The differentiated cells were incubated with (1 mg/ml) or without TG for 24 hr. (A) Cells were observed under a light microscope (Scale bars, 100 μm). (B) Viable cells were counted after conducting the trypan blue exclusion assay. Data are from three independent experiments. The number of viable differentiated THP-1 cells that were not treated with TG was set to 100%. Values are mean and standard error. P-values were determined by the Student's *t*-test.

IL-1β is a cytokine produced by activated macrophages and plays a key role during inflammatory reactions, cell proliferation/differentiation, and apoptosis in atherosclerosis (28-30). Interestingly, we found a gradual decrease in IL-1β expression in a TG dose-dependent manner (Fig. 2A and B) with a dramatic ~80% decrease in IL-1β expression beginning with 0.5 mg/ml TG ($P < 0.001$). Additionally, TG-treated cells exhibited a reduction in IL-1β expression in a time-dependent manner with a ~40% decrease following 12 hr of treatment ($P < 0.001$) (Fig. 2C). In the absence of TG treatment, IL-1β expression remained unchanged during the same time period, suggesting that the decrease in IL-1β expression was due to TG. It has been reported that VSMCs increase expression and secretion of IL-1β during apoptosis (22, 30). In stark contrast to these reports, our data showed a decrease in IL-1β expression during TG-induced death of THP-1 macrophages. Contrasting cellular responses can occur in different cells during apoptosis. This observation highlights the need to study a diverse array of cell types to fully understand the mechanism of TG-induced cell death in atherosclerosis.

The TG-induced decrease in IL-1β expression is mediated by p38 MAP kinase

We examined three signal transduction pathways mediated by p38 mitogen-activated protein kinase (p38 MAPK), protein kinase C (PKC), and RAF proto-oncogene serine/threonine-protein kinase (c-Raf) to identify the signaling pathway by which TG treatment downregulated IL-1β expression. p38 MAPK is activated in response to cytokines and stress associated with cell proliferation and apoptosis (31). PKC is a serine/threonine kinase that plays an important role in cell proliferation (32). c-Raf is a mitogen-activated protein kinase kinase kinase (MAP3K) that reg-

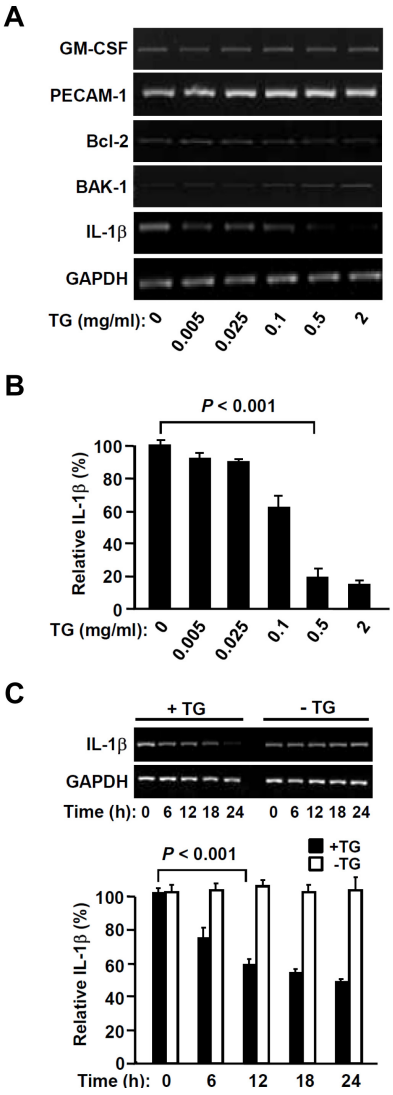

Fig. 2. Triglycerides (TG) downregulate interleukin-1β (IL-1β) expression in a dose- and time-dependent manner. (A) Differentiated THP-1 cells were treated with TG (0, 0.005, 0.025, 0.1, 0.5, and 2.0 mg/ml) for 24 hr. After total RNA extraction and cDNA synthesis, genes (GM-CSF, PECAM-1, Bcl-2, BAK1, and IL-1β) were amplified by PCR, and the PCR products resolved on a 1.5% agarose gel. GAPDH was used as the internal control. Data are from three independent experiments. (B) Densitometric analysis of IL-1β. The IL-1β expression level in cells not treated with TG was set to 100%. Data are from three independent experiments. (C) Differentiated THP-1 cells were incubated in the absence or presence of TG (1 mg/ml) for the indicated times (0, 6, 12, 18, and 24 hr). cDNA was synthesized and the IL-1β gene was amplified by PCR. PCR products were resolved on a 1.5% agarose gel (upper panel), and a densitometric analysis was performed (lower panel). GAPDH was used as the internal control. Data are from three independent experiments. IL-1β expression levels in cells treated with TG for 0 hr was set to 100%. Values are mean and standard error. P-values were determined by the Student's *t*-test.

ulates cell proliferation, differentiation, and apoptosis (33). THP-1-derived macrophages were incubated with select cell signaling inhibitors for p38 MAPK (SB203580), PKC (Ro317549), or c-Raf (GW5074), followed by treatment with TG. We found that the PKC specific inhibitor and the c-Raf inhibitor had no effect on TG-induced IL-1β downregulation. However, treatment with the p38 MAPK specific inhibitor prevented a decrease in IL-1β expression (Fig. 3). This result strongly suggests that the TG-induced decrease in IL-β expression was mediated by activation of the p38 MAPK pathway. Other cell signaling pathways may also be involved in TG-stimulated downregulation of IL-1β. Ongoing studies will likely shed light on the role of other cell signaling pathways.

In conclusion, we demonstrated that TG treatment of a human macrophage cell line resulted in decreased cell viability and a dramatic decrease in IL-1β expression via the p38 MAPK signal

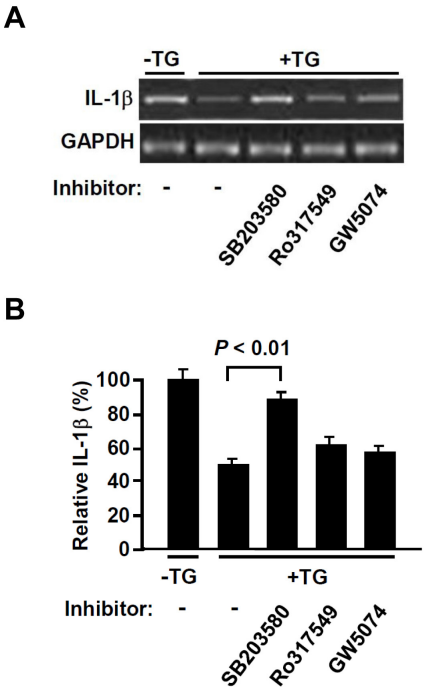

Fig. 3. Triglycerides (TG)-induced decrease in interleukin-1β (IL-1β) expression is mediated by p38 MAP kinase. Differentiated THP-1 cells were pre-incubated for 30 min with the p38 MAPK inhibitor (SB203580, 2.5 mM), PKC inhibitor (Ro317549, 500 nM), or the c-Raf inhibitor (GW5074, 100 nM) followed by treatment with or without TG (1 mg/ml) for 24 hr. cDNA was prepared, and the IL-1β gene was amplified by PCR. PCR products were resolved on a 1.5% agarose gel (upper panel), and a densitometric analysis was performed (lower panel). GAPDH was used as the internal control. Data are from three independent experiments. IL-1β expression levels in cells without inhibitor and without TG treatment were set to 100%. Values are mean and standard error. P-values were determined by the Student's *t*-test.

transduction pathway. We speculate that IL-1β downregulation may be associated with a cytotoxic effect of TG on macrophages. Further studies are warranted to understand the implication of these results in the context of atherosclerosis development.

MATERIALS AND METHODS

Materials

PMA was purchased from Sigma-Aldrich (St. Louis, MO, USA), and TG was provided by B. Braun Melsungen AG (Melsungen, Germany). Trypan blue stain solution and TRIzol for RNA isolation were obtained from Invitrogen (Carlsbad, CA, USA). The p38 MAPK (SB203580), PKC (Ro317549), and c-Raf (GW5074) inhibitors were purchased from EMD Biosciences (San Diego, CA, USA).

Cell culture and TG treatment

The THP-1 human acute monocytic leukemia cell line (ATCC, Manassas, VA, USA) was grown in RPMI media supplemented with 10% fetal bovine serum and penicillin-streptomycin and maintained at 37°C in a humidified atmosphere with 5% CO_2. To induce differentiation of THP-1 cells into macrophages, cells were seeded in 6-well plates at a density of 1×10^6 cells/well and treated with PMA (100 nM) for 48 hr. Thereafter, differentiated macrophages were incubated with the indicated concentrations of TG.

Trypan blue dye exclusion assay

Detailed methods were described previously (34). Differentiated macrophages were treated with or without TG (1 mg/ml) for 24 hr. The 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).

RNA extraction and reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from treated cells using TRIzol, 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 units of MMLV-RT for 50 min at 37°C and for 15 min at 70°C. Subsequent PCR amplification using 0.2 units of *Taq* polymerase (Cosmogenetech, Seoul, Korea) was performed in a thermocycler using specific primers. The primer sequences were as follows. GM-CSF: 5'-CTTCCTGTGCAACCCAG ATT-3' (forward), 5'-CTTCTGCCATGCCTGTATCA-3' (reverse), PECAM: 5'-CAGGGTGACACTGGACAAGA-3' (forward), 5'-GGAGCAGG ACAGGTTCAGTC-3' (reverse), Bcl-2: 5'-CATTTCCACGTCAACA GAATTG-3' (forward), 5'-AGCACAGGATTGGATATT CCAA-3' (reverse), BAK-1: 5'-GCCCAGGACACAGAGGAGGTT GGT-3' (forward), 5'-AAACTGGCCCAACAGAACCACACC-3' (reverse), IL-1β: 5'-AGCCATGGCAGAAGTACCT-3' (forward), 5'-CAGCTC TCTTTAGGAAGACA-3' (reverse), GAPDH: 5'-CGG GAAGCTT GTGATCAATGG-3' (forward), 5'-GGCAGTGATGGCATGGACT G-3' (reverse). GAPDH was used as the 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 the Gel Doc™ XR+ system. A densitometric analysis was performed using Image Lab software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

P-values were calculated using Student's *t*-test. A $P < 0.05$ was considered significant.

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