Regulatory effects of aggregated LDL on apoptosis during foam cell formation of human peripheral blood monocytes

Nobuhiko Kubo[a,*], Jiro Kikuchi[b,c], Yusuke Furukawa[b], Toshihiko Sakai[a], Hatsunori Ohta[a], Satsuki Iwase[d], Hisashi Yamada[d], Ikunosuke Sakurabayashi[a]

[a] *Clinical Laboratories, Omiya Medical Center, Jichi Medical School, 1-847 Amanuma, Omiya-shi, Saitama 330, Japan*
[b] *Division of Hemopoiesis, Institute of Hematology, Jichi Medical School, Tochigi 329-04, Japan*
[c] *Katsuta Research Laboratory, Hitachi Koki Co. Ltd., Ibaraki 312, Japan*
[d] *Department of Medicine (Aoto) and Institute of DNA Medicine, Jikei University School of Medicine, Tokyo 105, Japan*

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Abstract In order to investigate the mechanisms how modified lipoproteins enhance foam cell formation, we cultured peripheral blood monocytes with various stimulants and examined the effects of aggregated low-density lipoprotein (agLDL) on cell viability and lipid metabolism. AgLDL could completely inhibit phorbol ester-induced apoptosis, which was accompanied by intracellular cholesterol accumulation. Suppression of apoptosis-promoting proteases, ICE and CPP32, was observed in agLDL-treated cells. This indicates that agLDL accelerates foam cell formation through inhibition of apoptosis and enhancement of lipid accumulation in activated monocytes. By contrast, apoptosis was enhanced when monocytes were cultured with agLDL and M-CSF. Intracellular cholesterol accumulation was not significant in M-CSF treated cells. This suggests that M-CSF may act anti-atherogenic through apoptotic elimination of lipid-baring macrophages and enhanced lipid turnover. Our observation supports the novel hypothesis that regulation of apoptosis may play an important role in the development of atherosclerosis.

Key words: Atherosclerosis; Foam cell; Monocyte; Apoptosis; Low-density lipoprotein; CPP32

1. Introduction

Apoptosis is the cellular events of cell death that cause the elimination of certain stimulated cells to maintain tissue homeostasis [1]. It occurs during differentiation of lymphocytes, granulocytes, and also of monocytes [2]. Although many numbers of monocytes are produced daily and released into the blood stream [3], most of them are programmed to die via apoptosis in the absence of certain stimuli [4]. Defects in the apoptotic program may result in pathologic states such as development of monocytic leukemia [5] and severe tissue damage as observed in transgenic mice overexpressing granulocyte-macrophage colony stimulating factor (GM-CSF) [6]. Recently, it has been shown that several inflammatory cytokines such as interleukin-1 (IL-1) or IL-1 receptor antagonist are involved in the regulation of apoptosis of monocytes [4,5], and their down-stream effectors, including IL-1 converting enzyme (ICE) [7], CPP32 [8] and ICH-1 [9] were isolated.

Monocytes are now believed to play a pivotal role in the development of atherosclerosis [10]. Patrolling monocytes effectively recruit from the circulation to local inflammatory sites and can attach to injured endothelium of vessel walls under the influence of chemoattractants. Then, these monocytes migrate into the vessel walls and become macrophages if they can escape apoptotic cell death. Modified lipoproteins (oxidized LDL, acetyl LDL and aggregated LDL) are incorporated into macrophages and cause lipid accumulation [11]. This results in foam cell formation, which is a common feature of the earlier atherosclerotic lesion [12].

Although it is plausible that foam cell formation is regulated by the apoptotic program, to our knowledge there is no direct evidence indicating the relationship between these two processes. Certain stimulants, such as phorbol esters or M-CSF, have been shown to regulate both apoptosis [13] and lipid accumulation [14]. Therefore, using these reagents, it is possible to investigate how apoptosis is connected to intracellular lipid accumulation of monocytes leading to atherosclerosis.

Recently, several enzymes that mediate intracellular lipid metabolism have been characterized. Lysosomal acidic cholesteryl ester hydrolase (ACEH) releases free cholesterol (FC) from cholesteryl ester (CE) of incorporated lipoproteins [15]. Acyl-coenzyme A: cholesterol acyltransferase (ACAT) acts in cytoplasm and re-synthesizes CE from the released FC, and neutral CE hydrolase (NCEH) degrades accumulated CE in cytoplasm [16]. These enzymes play some roles in intracellular accumulation of CE, and are shown to be regulated by M-CSF during monocytic differentiation [17]. Recent investigations revealed that estradiol could increase NCEH activity in human monocytes, which may contribute to anti-atherogenic properties of the female gender [18]. Although the nature of NCEH is not fully understood, NCEH and hormone-sensitive lipase (HSL) are suspected to be the products of a single gene in macrophage [19].

In this study, we investigated the effects of agLDL on apoptosis of phorbol ester- or M-CSF-stimulated monocytes. We also examined the changes in expression of the molecules that modulate apoptosis (IL-1β, ICE, CPP32 and ICH-1) and lipid metabolism (ACEH, ACAT and HSL) in order to know how apoptotic events and cholesterol accumulation are interrelated and implicated in the development of atherosclerosis.

2. Materials and methods

2.1. Reagents

All chemicals including phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise and were of the highest purity available. Recombinant human M-CSF with a specific activity of 2×10^5 U/mg was provided by Morinaga Milk Industry Co. Ltd. (Tokyo, Japan).

*Corresponding author. Fax: (81) 48-648-5188

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2.2. Preparation of LDL

LDL was isolated from the serum of normolipidemic donors by sequential ultracentrifugation at densities between 1.006 and 1.065 g/ml in a ultracentrifuge (model CP100-α, Hitachi Koki Co., Ibaraki, Japan) and was dialyzed at 4°C against phosphate buffer solution (PBS; pH 7.5) [20]. Aggregated LDL was made by vortexing for 60 s in Eppendorf tubes as previously described [21].

2.3. Isolation of monocytes and culture

Normal human monocytes were isolated from the peripheral blood of healthy volunteers by counterflow centrifugal elutriation (SRR6Y elutriation system, Hitachi Koki Co. Ltd., Ibaraki, Japan) as described elsewhere [22]. Monocyte-enriched fractions (>90% purity) were resuspended at 3×10^6 cells/ml in Dulbecco's modified Eagle's medium (Life Technologies Inc., Grand Island, NY) and cultured in the presence of autologous serum (0.2%). Where indicated, PMA and M-CSF were added at the final concentration of 10 ng/ml and 50 ng/ml, respectively, which was determined as optimal in pilot experiments.

2.4. Assessment of apoptosis

The proportion of apoptotic cells was quantified by flow cytometric analysis and DNA electrophoresis as previously described [23]. The percentage of subdiploid fractions was calculated using FACScan/CellFIT system (Becton-Dickinson, San Jose, CA).

2.5. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR analysis was carried out as previously described [23]. Total cellular RNA from 1×10^5 cells was reverse transcribed into cDNA and amplified by PCR for 35 cycles with the following primer pairs (predicted molecular sizes are shown in parentheses): IL-1β, (1) 5′-CTTCATCTTTGAAGAAGAACCTATCTTCTT-3′ and (2) 5′-AATTTTTGGGATCTACACTCTCTCCAGCTGTA-3′ (323 bp) [24]; ICE, (1) 5′-AGGACAACCCAGCTATGCCCACAT-3′ and (2) 5′-CTTCGGTTTGTCCTTCAAACTT-3′ (483 bp) [7]; CPP32, (1) 5′-CTCAGTGGATTCAAAATCCAT-3′ and (2) 5′-CTGTCTGTCTCAATGCCACAG-3′ (510 bp) [8]; ICH-1, (1) 5′-CCAGCTGGCATATAGGTTGCAGT-3′ and (2) 5′-TCAACCCCAAGATCAGTCTCA-3′ (488 bp) [9]; ACAT, (1) 5′-GAGGAAGATGAAGACCAGAGAAAC-3′ and (2) 5′-TGTTCTGATGAGGTCCAGTTCAAG-3′ (359 bp) [25]; ACEH, (1) 5′-ACAGATCCCTGAGCTGGCTA-3′ and (2) 5′-TCCAGACTGCAGTCGGCACA-3′ (467 bp) [15]; HSL, (1) 5′-GCCAGAAAAGACACCCATAGCCCA-3′ and (2) 5′-GCCAGCATTGAGACAAAGACATGT-3′ (324 bp) [26]; GAPDH (glyceraldehyde-3-phosphate dehydrogenase), (1) 5′-CCACCCATGGCAAATTCCATGGCA-3′ and (2) 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (600 bp) [27].

2.6. Measurement of intracellular cholesterol

Cells were washed 3 times with PBS, and were dissolved in PBS

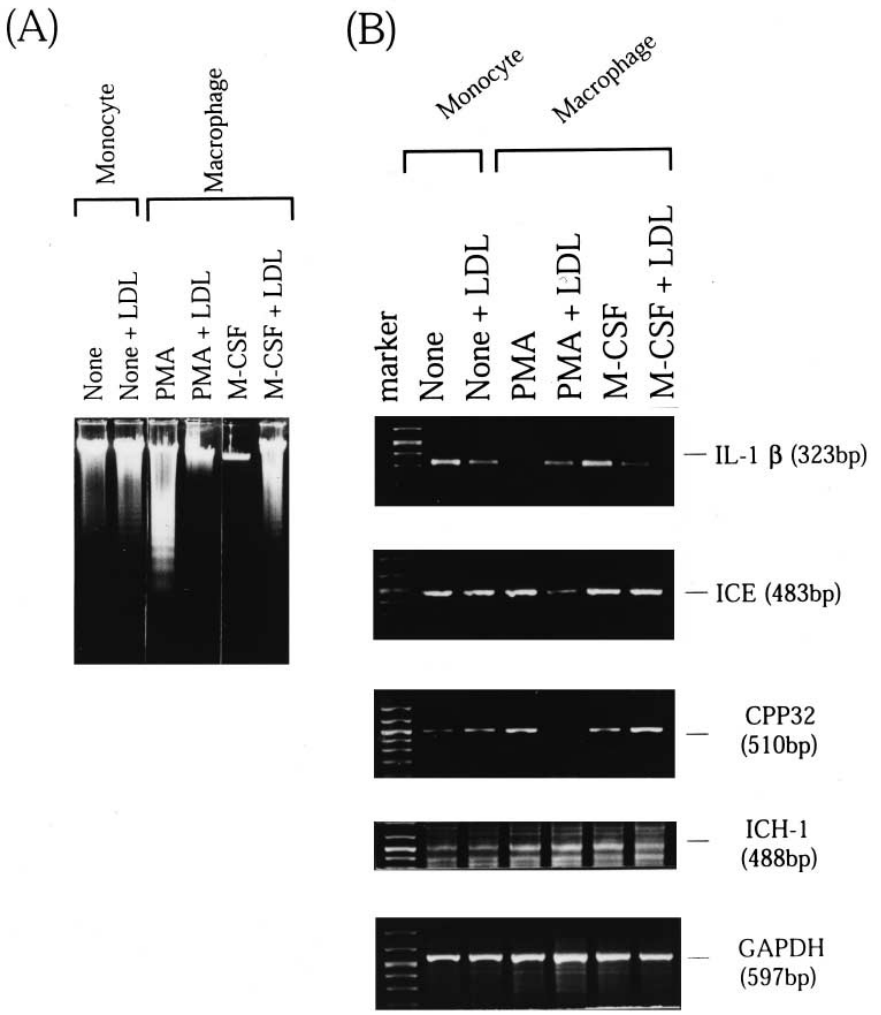

Fig. 1. Effects of agLDL on apoptosis and expression of apoptosis-related genes in monocytes. Human peripheral blood monocytes were cultured with no stimulants, 10 ng/ml of PMA or 50 ng/ml of M-CSF in the absence (None, PMA and M-CSF) or presence of agLDL (+LDL). A: DNA was isolated after 48 h and subjected to agarose gel electrophoresis. B: RNA was isolated after 12 h and subjected to semiquantitative RT-PCR analysis for IL-1β, ICE, CPP32, ICH-1 and GAPDH mRNA expression. Bio Marker Low (Bio Ventures, Inc., Murfreesboro, TN) was used as a molecular size marker (marker).

containing Triton-X (0.3%) and cholic acid (0.3%). Thereafter, cholesterol concentration of the solution was measured according to the standard method using cholesterol oxidase with minor modifications [28]. Cholesteryl ester hydrolase was included in the reaction mixture for the determination of total cholesterol (TC). Free cholesterol (FC) level was determined in the assay without cholesteryl ester hydrolase. Cholesteryl ester concentration was calculated as TC minus FC.

2.7. Other assays

Protein concentration was measured by the microprotein assay (BioRad Co., Richmond, CA) with bovine serum albumin as a standard. Oil red staining was performed with 3 mg/ml of oil red in 60% ethanol as described previously [11]. All experiments were carried out for a minimum of 3 times in triplicate or quadtriplicate. Results were expressed as mean ± SD and statistically evaluated by paired Student's *t*-test.

3. Results

3.1. Effects of aggregated LDL on apoptosis of monocytes

Human peripheral blood monocytes were isolated in a nascent state by centrifugal elutriation, and cultured in the absence or presence of various stimulants. After 48 h of the culture, the cells were harvested for the evaluation of apoptosis by flow cytometry and DNA electrophoresis. When monocytes were cultured without any stimulants, subdiploid DNA fractions, which correspond to DNA fragmentation, were observed in approximately 25% of the cells on propidium iodide staining. Consistent with the result of flow cytometric analysis, agarose gel electrophoresis revealed moderate level of oli-

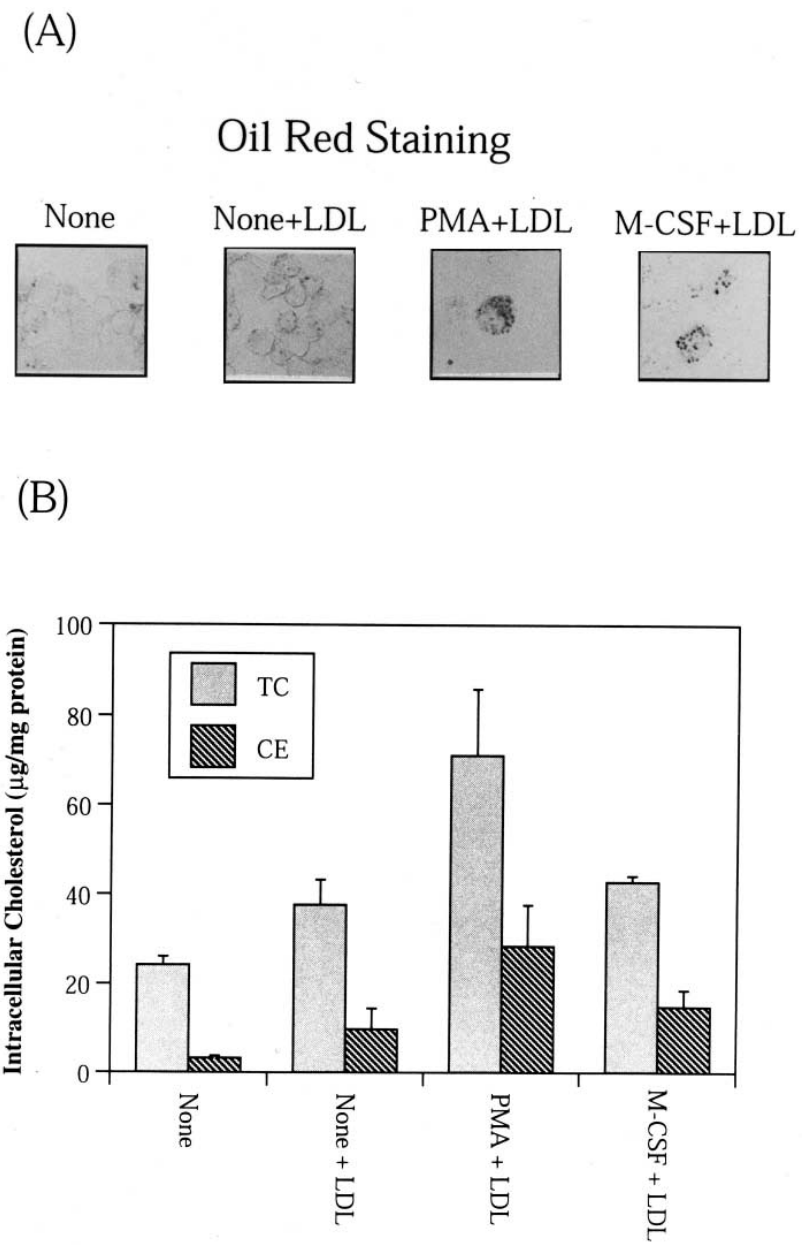

Fig. 2. Lipid accumulation in unstimulated and stimulated monocytes. Monocytes were cultured with various stimulants as described in Fig. 1 legend. A: The cells were harvested after 48 h, and subjected to oil red staining on cytospin slides. Original magnification: ×400. B: Intracellular cholesterol (TC; total cholesterol, CE; cholesteryl ester) was measured by the enzyme-based assay methods. Mean ± SD (bar) of three independent experiments was shown.

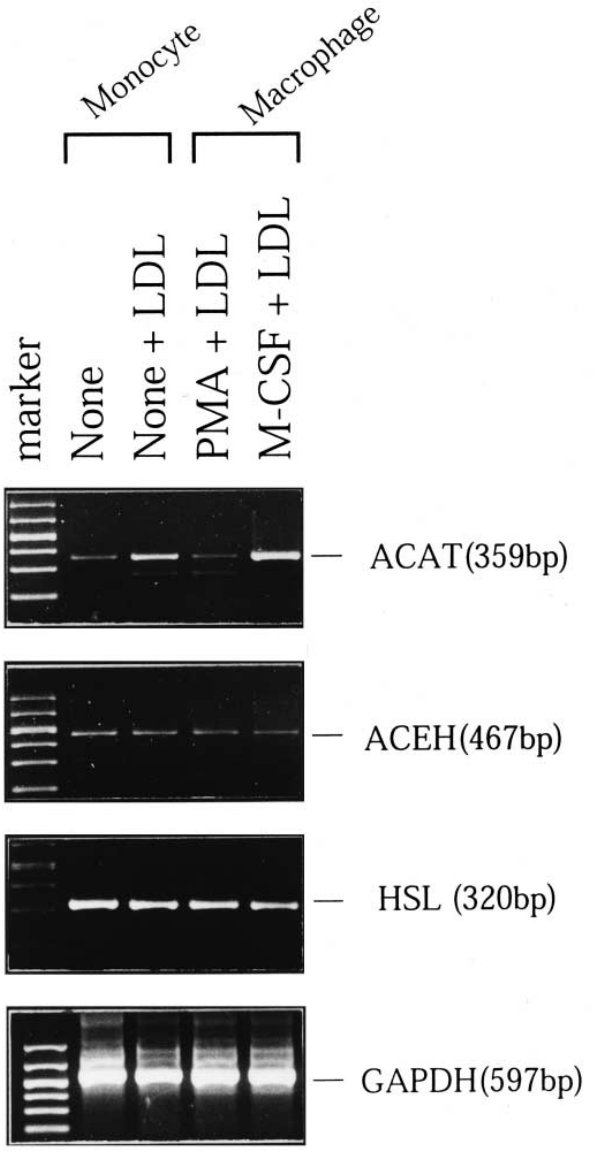

Fig. 3. Expression of the genes involved in lipid metabolism during foam cell formation. Total cellular RNA was isolated as described in Fig. 1 legend, and expression of ACAT, ACEH, HSL and GAPDH mRNAs was examined by semiquantitative RT-PCR according to the standard method. Bio Marker Low was used as a molecular size marker (marker).

gonucleosomal-length DNA fragmentation (Fig. 1A, None). Activation of monocytes with PMA resulted in marked enhancement of apoptosis, i.e. the proportion of subdiploid fractions was increased to 46 ± 8% and DNA fragmentation was significantly enhanced (Fig. 1A, PMA). In contrast, M-CSF could inhibit spontaneous apoptosis of monocytes as indicated by the disappearance of DNA fragmentation (Fig. 1A, M-CSF). The percentage of subdiploid fractions was also decreased to 19 ± 7% after 48 h of the culture with M-CSF.

With this culture system, we investigated the effects of agLDL on apoptosis of activated and non-activated monocytes. AgLDL did not affect spontaneous apoptosis of monocytes at the concentrations between 0.1 and 1.0 mg/ml (the result with 0.5 mg/ml was shown in Fig. 1A, None+LDL). On the other hand, agLDL could inhibit PMA-induced apoptosis of monocytes. DNA fragmentation was almost completely suppressed by the addition of agLDL (Fig. 1A, PMA+LDL) and the proportion of subdiploid fractions was reduced to 30 ± 17% ($P < 0.05$ vs. PMA alone). On the contrary, the inhibitory effect of M-CSF on apoptosis was abrogated by agLDL. AgLDL induced typical DNA ladder in M-CSF-treated monocytes (Fig. 1A, M-CSF+LDL) and increased the percentage of subdiploid fractions to 26 ± 2% ($P < 0.05$ vs. M-CSF alone) on flow cytometric analysis.

3.2. *Expression of apoptosis-related genes in monocytes treated with agLDL*

To elucidate the mechanisms how LDL modulates apoptosis of activated monocytes, we investigated expression of some apoptosis-related genes (IL-1β, ICE, CPP32 and ICH-1) by semiquantitative RT-PCR. We chose this method because only relatively small amounts of RNA could be obtained from dying cells, and many genes including a ubiquitously expressed control (GAPDH in this study) could be detected simultaneously in the same sample [23]. Any apoptosis-related gene was not expressed in monocytes just after the isolation, whereas GAPDH mRNA was readily detectable (data not shown). Fig. 1B shows the representative result with samples isolated after 12 h of the culture. In consistent with the induction of spontaneous apoptosis, ICE, CPP32 and ICH-1 mRNA transcripts, which encode cysteine proteases with properties of mediating apoptosis, were induced during culture without any stimulants (Fig. 1B, None). Increased expression of IL-1β, which acts on monocytes to prevent apoptosis [4,5], was also observed. AgLDL did not affect expression of these genes in unstimulated monocytes (Fig. 1B, None+LDL). In contrast, both CPP32 and ICE mRNAs were up-regulated in PMA-activated monocytes, whereas IL-1β expression was remarkably suppressed (Fig. 1B, PMA). This imbalance between apoptosis inducer (CPP32, ICE) and protector (IL-1β) may at least in part contribute to marked enhancement of apoptosis in PMA-treated cells. AgLDL could dramatically modulate the effects of PMA on expression of these genes, i.e. the amounts of CPP32 and ICE mRNA transcripts were significantly decreased in cells treated with PMA and agLDL (Fig. 1B, PMA+LDL). Similarly, PMA-induced suppression of IL-1β was reversed by the addition of agLDL. These changes may explain why agLDL can suppress PMA-induced apoptosis. M-CSF did not modulate the expression of these genes, suggesting that its anti-apoptotic effects might be mediated through distinct mechanisms. However, agLDL could significantly increase CPP32 mRNA expression and also decreased IL-1β mRNA expression in M-CSF-treated monocytes (Fig. 1B, M-CSF+LDL). This is compatible with apoptosis-inducing properties of agLDL in M-CSF-treated cells. Expression of ICH-1 and GAPDH, an internal control, was stable in these samples.

3.3. *Lipid accumulation in agLDL-treated monocytes*

Next, we examined the levels of intracellular lipid accumulation in monocytes cultured with agLDL in the absence or presence of the stimulants (PMA or M-CSF). After 48 h of the culture, cells were stained with oil red on cytospin specimens. As shown in Fig. 2A, lipid accumulation was most striking in monocytes cultured with PMA (PMA+LDL). It was weak in M-CSF-treated cells (M-CSF+LDL). No significant staining was observed in those cultured with agLDL alone (None+LDL). Then, intracellular cholesterol levels

were determined by enzyme-based assay methods (Fig. 2B). The amount of total cholesterol (TC) in cells treated with PMA and LDL was significantly higher than that of agLDL alone (70.9 ± 14.7 mg/mg protein vs. 37.6 ± 5.8 mg/mg protein, $P < 0.05$). In contrast, the difference in TC levels between cells stimulated with M-CSF+agLDL and those with agLDL alone was not significant (42.7 ± 1.3 mg/mg cell protein vs. 37.6 ± 5.8 mg/mg cell protein, $P > 0.05$). The pattern of intracellular cholesteryl ester (CE) accumulation was similar to that of TC, i.e., PMA+LDL (28.2 ± 9.4 mg/mg cell protein) but not M-CSF+LDL (14.8 ± 3.9 mg/mg cell protein) showed a significant level of deposits as compared to agLDL alone (10.1 ± 4.5 mg/mg cell protein). Either PMA alone or M-CSF alone could not enhance the accumulation of intracellular TC and CE (data not shown).

3.4. *Expression of the genes involved in lipid metabolism (ACAT, ACEH and HSL) in monocytes during foam cell formation*

In an attempt to clarify the mechanisms of intracellular cholesterol accumulation during foam cell formation, we carried out RT-PCR analysis for some genes involved in lipid metabolism (ACAT, ACEH and HSL) using RNA from monocytes cultured as described above. As depicted in Fig. 3, ACAT mRNA was apparently up-regulated by M-CSF in the presence of agLDL, whereas it was not changed by other stimulants such as agLDL alone or PMA+agLDL. On the other hand, expression of ACEH and HSL mRNA transcripts was constitutive as compared to that of GAPDH, and unaffected by any stimulants tested (Fig. 3).

4. Discussion

In this study, we investigated the role of apoptosis in foam cell formation from activated monocytes. First, in order to examine how modified lipoproteins modulate apoptotic cell death, we exposed human peripheral blood monocytes to aggregated LDL in the absence or presence of various stimulants such as PMA and M-CSF. Activation of monocytes with PMA resulted in massive apoptotic cell death as recently reported [13]. When agLDL was added to the culture with PMA, apoptosis was almost completely inhibited, which was accompanied by intracellular cholesterol accumulation. This suggests that inhibitory effect of agLDL on activation-induced apoptosis may contribute to differentiation of monocytes into macrophages and subsequent foam cell formation.

When monocytes were cultured with M-CSF, spontaneous cell death was significantly inhibited in consistent with the previous observation [29]. Interestingly, the addition of agLDL reversed anti-apoptotic effect of M-CSF, suggesting that M-CSF, as opposed to PMA, could prevent foam cell formation of monocytes through induction of apoptosis. In addition, intracellular cholesterol was not significantly accumulated in M-CSF-treated cells. It has been reported that M-CSF stimulates the clearance of lipoproteins in rabbits [30]. It also stimulates the uptake and degradation of acetylated LDL, cholesterol esterification, and cholesterol efflux in human macrophages [17]. In sum, this cytokine is thought to possess protective effects against atherosclerosis. Our present finding is in line with these observations, and provides the first evidence that M-CSF may also act anti-atherogenic through the elimination of lipid-baring macrophages by apoptosis.

Recent investigation demonstrated that execution of apoptotic process involves redundant activation of several proteases [31]. These proteases are considered to be mammalian homologs of ced-3 of *Caenorhabditis elegans*, and share a common sequence, QACRG, which contains the active-site cysteine. They are now subdivided into three groups (ICE-, CPP32- and ICH-1-related protease family) and functional analysis, including identification of their substrates, are being progressed [31]. In this study, we examined the expression of these apoptosis-promoting proteases during foam cell formation of monocytes, and found that levels of ICE and CPP32 but not ICH-1 were dramatically changed in accordance with the modulation of apoptosis by agLDL. Given that activation of these proteases is regulated by extracellular signals such as Fas [32], it is likely that LDL can also directly modify the expression of these molecules. It seems interesting to investigate its signaling pathways in LDL-treated macrophages. In this context, it is of note that CPP32 may participate in up-regulation of sterol import by activating sterol response element-binding proteins to induce transcription of genes involved in sterol metabolism [33]. This suggests that, in concert with our findings, CPP32 is a key molecule to further clarify the relationship between apoptosis and lipid accumulation during development of atherosclerosis.

In addition, we found that IL-1β mRNA expression was also regulated by agLDL in activated monocytes. Since IL-1β is known to suppress apoptosis of monocytes [4] and is a major substrate of ICE [7], the balance between IL-1β and ICE-like proteases may also be a determinant of cell fate. Indeed, relative increase in IL-1β over ICE was observed when apoptosis was suppressed in PMA-activated monocytes by agLDL, and vice versa in cells treated with M-CSF and agLDL. Moreover, agLDL-induced IL-1β production may also contribute to the formation of atherosclerotic lesions, since IL-1β promotes proliferation of smooth muscle cells [10].

Accumulation of CE in PMA/agLDL-stimulated cells could not be explained by the changes in specific gene expression. For example, PMA-induced increase of ACAT was abrogated in the presence of agLDL. Furthermore, the amounts of ACEH and HSL were comparable between cells stimulated by PMA alone and those with PMA and agLDL. Therefore, down-regulation of NCEH activity, other than HSL, may be responsible for lipid accumulation during foam cell formation. Previous study demonstrated that M-CSF could increase NCEH activity in cultured macrophages, suggesting that NCEH is also important for M-CSF to act anti-atherogenic [14]. Further characterization of the NCEH activity is required for better understanding of the mechanisms of foam cell formation.

In summary, our observation supports novel hypothesis that inhibition of apoptotic elimination of lipid-baring macrophages by modified lipoproteins, along with enhanced lipid accumulation, may contribute to the development of atherosclerosis.

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