Original Article

The Potential Role of Macrophage Migration Inhibitory Factor on the Migration of Vascular Smooth Muscle Cells

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***Aim*: Macrophage migration inhibitory factor (MIF) is known as a pro-inflammatory cytokine that regulates a broad spectrum of inflammatory reactions. MIF is expressed in vascular smooth muscle cells (VSMCs), and inhibition of the progression of atherosclerosis was observed in MIF-deficient atherosclerotic mice. However, the functional role of MIF in VSMCs has not been elucidated. The aim of this study was to investigate the role of MIF on the migration of VSMCs.**

***Methods*: Cultured rat A10 cells, derived from rat embryonic aortic smooth muscle cells, were stimulated with oxLDL, and the effect of MIF knockdown on oxLDL-mediated migration of A10 cells was analyzed.**

***Results*: Intracellular MIF content was significantly increased and a marked increase of MIF concentration was observed in the supernatant of A10 cells treated with oxLDL. The migration of A10 cells was significantly accelerated by the stimulation of recombinant MIF in a dose-dependent manner. Notably, knockdown of intracellular MIF by siRNA abolished oxLDL-induced migration of A10 cells.**

***Conclusion*: These findings suggest that MIF acts on the migration of VSMCs in an autocrine and paracrine fashion. MIF appears to be a novel target for the prevention of cardiovascular events.**

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Key words; MIF, Vascular smooth muscle cells, Migration, Atherosclerosis

Introduction

Macrophage migration inhibitory factor (MIF) was first described as a product of activated T cells and previous publications led to the recognition that MIF plays a potential role in regulating a broad spectrum of biological events, including inflammation and immune regulation[1-4]. Importantly, the expression and secretion of MIF is not restricted to immune cells[5]. Vascular endothelial cells (VECs) and smooth muscle cells (VSMCs) were found to produce MIF[6, 7]. Recent publications have suggested that MIF plays a potential role in the progression of atherosclerosis[6].

The pathogenesis of atherosclerosis is characterized by a chronic inflammatory response of the arterial wall to injury[8]. Monocytes and macrophages have been shown to play a central role in the development of atherosclerotic plaques[8]. An early event in atherosclerosis is monocyte adherence to activated endothelial cells[9]. After transmigrating across the endothelial cell layer, monocytes mature into macrophages which phagocytose lipids to become foam cells forming an early fatty streak. Of note, the early stage of atherosclerosis is also characterized by the formation of oxidized low-density lipoprotein (oxLDL) in the vascular wall. According to previous findings, the expression of MIF in macrophages is markedly increased in response to oxLDL[10]. oxLDL further increases the secretion of MIF by macrophages and MIF stimulates oxLDL uptake in an autocrine and paracrine fashion. These data indicate that MIF has in potential role in acceleration of the formation of foam cells.

The evolution of atherosclerosis is characterized by VSMC expansion and activation. These phenotypically modified VSMCs constitute a major part of restenosis[11] and release proinflammatory cytokines, such as tumor necrosis factor alpha (TNFα, interleukin-6 (IL-6) and monocyte chemoatractant protein-1

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(MCP-1)[12, 13]. These cytokines amplify the inflammatory response and serve to sustain the inflammation in advanced plaque. Moreover, the migration of VSMCs from the media to the intima is a key process in the development of both atherosclerosis and restenosis. Interestingly, increased production of MIF has been observed in VSMCs during the progression of atherosclerotic plaque evolution in humans and in a hypercholesterolemic rabbit model[6, 14]. Recent *in vivo* studies have provided evidence of the role of MIF in accelerated atherosclerosis and re-stenosis[15]. Pan *et al.* demonstrated that $MIF^{-/-}$/LDL-$R^{-/-}$ double knockout mice had reduced intimal thickening in the aortic arch and the atherosclerotic lesion area in the abdominal aorta, accompanied by diminished VSMC proliferation and proteolytic capacity[16]. These observations suggested that MIF contributes to advanced complicated lesion formation in atherosclerosis; however, the functional role of MIF in the migration of VSMCs has not been elucidated.

In the present study, we obtained direct *in vitro* evidence that MIF acts as a mediator of the migration of VSMCs by studying the effects of MIF on the ox-LDL-mediated migration of VSMCs.

Methods

Materials

Rabbit anti-MIF polyclonal antibody, and recombinant human MIF and A10 cell lines, derived from rat embryonic aortic smooth muscle cells, were generous gifts from Dr. Nishihira (Hokkaido University). Anti-actin antibody was obtained from Sigma (St. Louis, MO).

Cell Culture

Rat A10 cells were cultured in DMEM supplemented with 10% heat-inactivated FCS at 37℃ under 5% CO_2, and subcultured every 3 days. DMEM supplemented with 1%FCS was used in the secretion and expression assay of MIF. Serum-free DMEM was used in the migration assay to reduce the effect of serum. Cell viability in serum-free medium and 1%FCS-containing medium was analyzed by trypan blue exclusion. No significant loss of viable A10 cells was observed under these conditions.

Preparation of Oxidized LDL

LDL (d=1.019-1.063) was isolated from fresh normal human plasma by ultracentrifugation. LDL was oxidized by incubating LDL (200 μg of protein/mL) with 5 μM $CuSO_4$ in phosphate-buffered saline without EDTA for 20 h at 37℃[10, 17]. The degree of oxidation was assessed by the increase of electro-negative mobility in an agarose gel. In addition, the levels of oxidized lipids were evaluated by measuring thiobarbituric acid-reactive substance (TBARS) levels.

ELISA Analysis of MIF

For the quantification of MIF, we performed a one-step sandwich ELISA consisting of a solid phase anti-MIF antibody and horseradish peroxidase-conjugated anti-MIF antibody using IDLISA Rat/Mouse MIF (Sapporo Immuno Diagnostic Laboratories, Sapporo, Japan).

Immunoblot Analysis

Cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris pH7.5) containing protease inhibitors (Complete, Mini, EDTA-free, Roche Diagnostics Co, Indianapolis, IN), and disrupted by aspiration through a 21-gauge needle. After incubation on ice for 30 min and micro-centrifugation for 10 min, the supernatants were collected and protein concentration was determined by DC Protein Assay (Bio-Rad, Hercules, CA), according to the manufacturer's recommendations. Equal amounts of cellular proteins were fractionated on SDS gels, transferred to polyvinylidine difluoride membranes (Millipore, Bedford, MA), and analyzed by Western blotting. The expression of MIF was revealed with an anti-MIF polyclonal antibody in combination with peroxidase-labeled secondary antibody and blots were developed by Super Signal West Femto Maximum Sensitivity Substrate (Pierrce, Rockford, IL). Blots were reprobed with anti-actin antibody.

Migration Assay

Migration of A10 cells was measured by the migration of cells through a Matrigel-coated Chemotaxicell chamber (KURABO, Osaka, Japan). After treatment with MIF siRNA or control siRNA, cells were cultured in DMEM containing 1%FCS with or without oxLDL for 12 h. Cells were plated in the upper chamber and allowed to migrate for 12 h. Noninvasive cells were removed from the upper chamber with a cotton swab, and migrating cells adhering to the underside of the filter were fixed, and stained with Diff Quick solution. Ten random fields per filter were counted. All experiments were performed independently and in triplicate.

Transfection with siRNA

The RNAi technique is used to down-regulate the expression of specific genes in living cells by introducing a homologous double-stranded RNA, and 21-base

siRNAs are potent mediators of the RNAi effect in mammalian cells. The nucleotide sequences of dsRNA and complimentary dsRNA for mouse mRNA were 5'-CCGCAACUACAGUAAGCUGdTdT-3' and 5'-CAGCUUACUGUAGUUGCGGdTdT-3', respectively. As a control RNA duplex, 5'-GCGCGCUUUGUAGGAUUCGdTdT-3' and 5'-CGAAUCCUACAAAGCGCGCdTdT-3' were used. A10 cells in culture dishes (60 mm in diameter) containing DMEM supplemented with 1%FCS were transfected with either MIF siRNA or control RNA duplex using siFECTOR (B-Bridge, Sunnyvale, CA) according to the manufacturer's protocol.

Statistical Analysis

Significance of difference was determined by one-way analysis of variance (ANOVA) following Tukey's test for the comparison of individual treatment groups. $P < 0.05$ was considered statistically significant.

Results

Effect of oxLDL on MIF Secretion by A10 Cells

oxLDL has been reported to induce macrophage and endothelial MIF expression[10, 14]; therefore, we evaluated the effect of oxLDL on MIF production and release by VSMCs. The preparation of oxLDL was described in the Methods. TBARS levels were increased in oxLDL compared to native LDL (44.0 versus 3.2 nmol MDA/mg LDL protein), and the increased oxidation of LDL was also confirmed by the increase of electro-negative mobility in an agarose gel (**Fig. 1A**). A10 cells were cultured in DMEM containing 1%FCS for 24 h and cells were stimulated with 50 μg/mL of oxLDL. The concentration of MIF in the supernatant was measured by ELISA, as described in Methods. As compared with control and native LDL, a marked increase of MIF content was observed in the supernatant of oxLDL-treated A10 cells (**Fig. 1B**), and the effect of oxLDL on MIF release was observed in a dose-dependent manner (**Fig. 1C**).

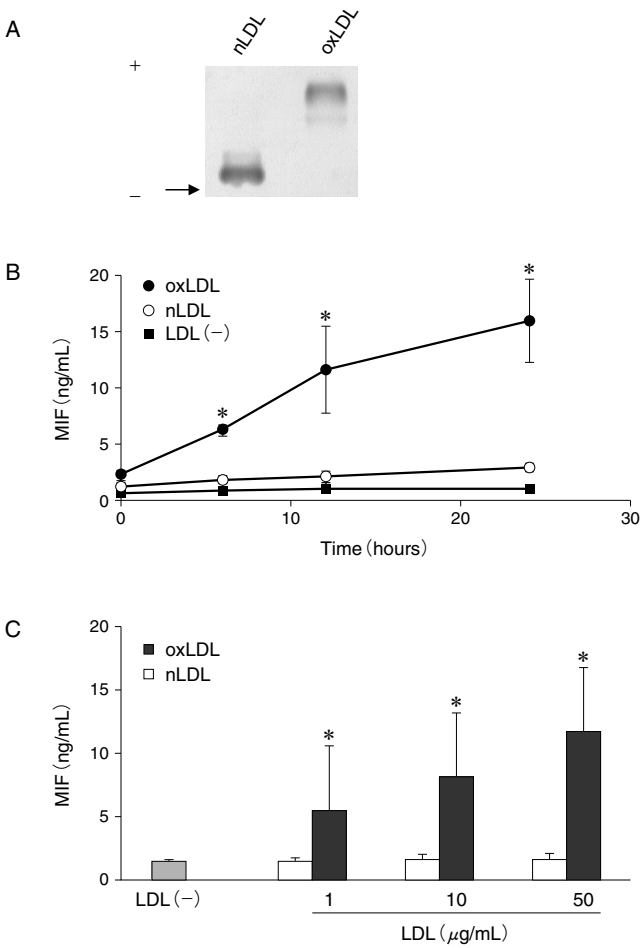

Fig. 1. MIF secretion by A10 cells induced by oxLDL.

A10 cells were incubated with DMEM supplemented with 1%FCS for 24 h, following the stimulation of oxLDL. The concentration of MIF in supernatant was determined by ELISA, as described in Methods. Electro-negative mobility of oxLDL and native LDL in an agarose gel (A). oxLDL induced a time-(B) and dose (C)-dependent increase of MIF content in the supernatant of A10 cells. Values represent the mean ± SD of three experiments. $^*p < 0.05$ versus control value.

Effect of oxLDL on MIF Protein in A10 Cells

The effect of oxLDL on the expression of MIF protein in A10 cells was examined by immunoblotting using anti-MIF antibody. As shown in **Fig. 2**, the expression of MIF protein was markedly increased by stimulation of oxLDL at 6, 12 and 24 h compared to the stimulation of native LDL.

Effect of Recombinant MIF on the Migration of A10 Cells

To evaluate the functional role of MIF induced by oxLDL in A10 cells, we examined the migration of A10 cells in the presence of recombinant MIF using an established protocol. The increase in the migration of A10 cells was significantly associated with the concentration of MIF ($p < 0.001$, ANOVA). MIF enhanced the migration of A10 cells in a dose-dependent manner (**Fig. 3**).

Effect of Inhibition of MIF Expression in A10 Cells

Since the potential role of oxLDL in the migration of vascular endothelial cells (VECs) has been shown in previous reports, it seemed reasonable to hypothesize that oxLDL-induced release of MIF is asso-

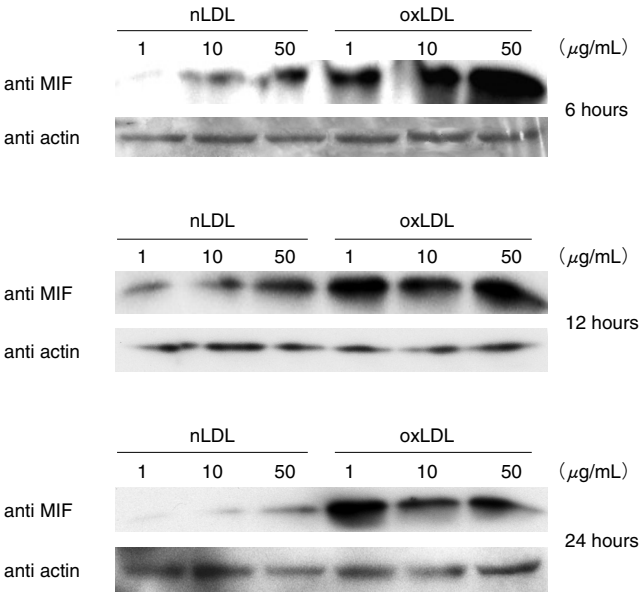

Fig. 2. Expression of MIF protein in A10 cells in response to oxLDL.

A10 cells were incubated with DMEM supplemented with 1%FCS for 24 h and cells were exposed to oxLDL or native LDL. The expression of MIF protein was determined by immunoblotting, as described in Methods.

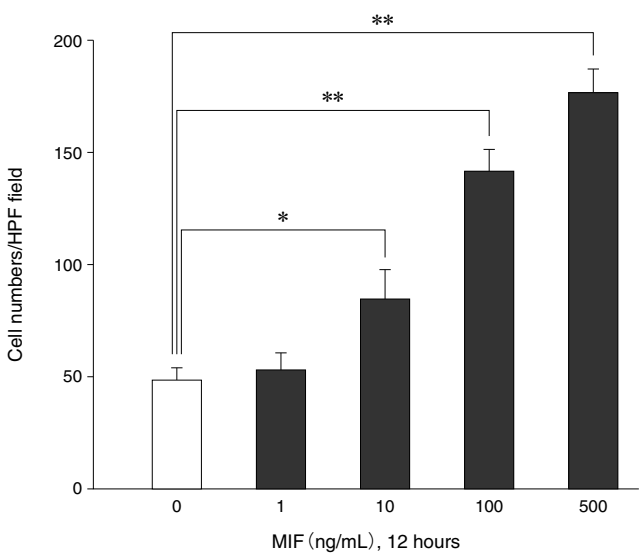

Fig. 3. Migration of A10 cells by the stimulation of recombinant MIF.

MIF-mediated migration of A10 cells was determined by migration assay, as described in Methods. Values represent the mean ± SD of three experiments. $^{*}p<0.05$, $^{**}p<0.01$.

ciated with the migration of A10 cells in an autocrine and paracrine fashion. To investigate this hypothesis, we examined the effect of MIF knockdown on the

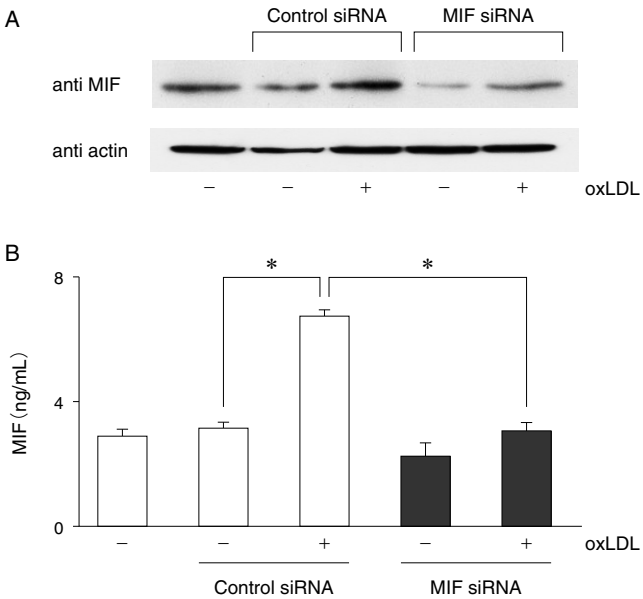

Fig. 4. Inhibitory effect of MIF siRNA on the expression of MIF.

A10 cells were treated with siRNA for MIF or control siRNA, and the oxLDL-mediated induction of MIF protein in A10 cells was determined by immunoblotting (A). The oxLDL-mediated secretion of MIF by A10 cells was determined by ELISA (B). Values represent the mean ± SD of three experiments. $^{*}p<0.01$.

oxLDL-mediated migration of A10 cells. First, we investigated the effect of siRNA on the expression of MIF protein by immunoblotting. As shown in **Fig. 4A**, the expression of MIF in A10 cells treated with oxLDL was markedly reduced by the administration of MIF siRNA. The amount of actin was measured as a control. Consistent with this result, oxLDL-mediated secretion of MIF by A10 cells was significantly inhibited by the administration of MIF siRNA (**Fig. 4B**). Thus, we confirmed the inhibition of oxLDL-dependent induction of MIF in A10 cells by MIF siRNA. Finally, we studied the effect of MIF siRNA on the oxLDL-induced migration of A10 cells. The cells were treated with MIF siRNA or control siRNA for 48 h, following stimulation with oxLDL (50 μg/mL) for 12 h. As shown in **Fig. 5**, MIF siRNA treatment led to the reduction of oxLDL-mediated migration of A10 cells, whereas control siRNA did not affect the upregulation of A10 cell migration induced by the stimulation of oxLDL.

Discussion

oxLDL has been reported to induce macrophage and endothelial MIF expression[10, 14]. In the present

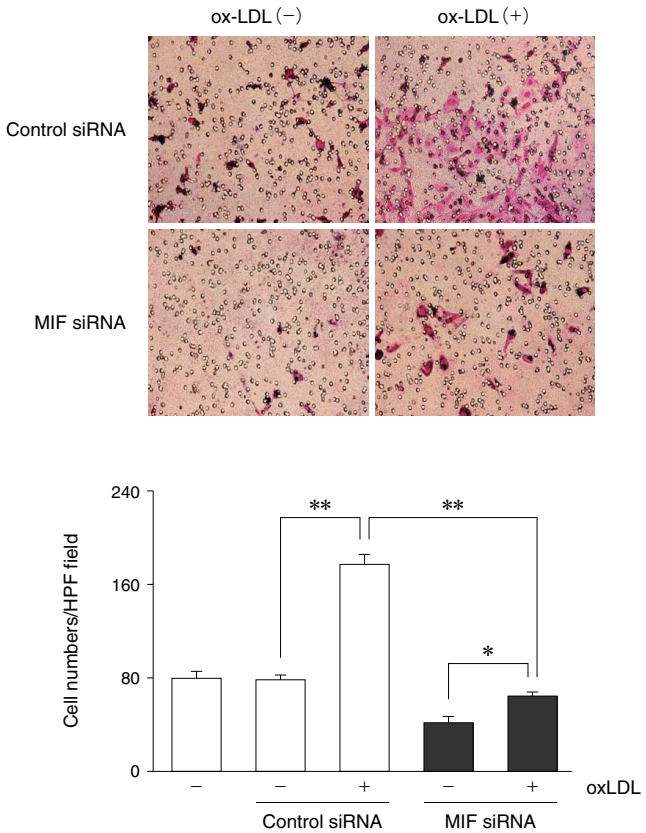

Fig. 5. Inhibitory effect of MIF siRNA on the oxLDL-induced migration of A10 cells.

A10 cells were treated with siRNA for MIF or control siRNA and the effect of oxLDL on the migration of A10 cells was determined. Upper panel represents cells migrated through the pores of membranes. Cells were stained with Diff Quick solution. Lower panel represents quantitative analysis, as described in Methods. Values represent the mean ± SD of three experiments. $^{*}p<0.05$, $^{**}p<0.01$.

study, we demonstrated that the expression and secretion of MIF by A10 cells was increased by the stimulation of oxLDL. Therefore, oxLDL seems to be a potent stimulator for the induction of MIF during the progression of atherosclerosis. We also observed that recombinant MIF enhanced the migration of A10 cells. The range of MIF concentration in this study was 1-500 ng/mL, and a significant effect of MIF on the migration of A10 cells was observed at concentrations of MIF that are within the range for those reported under pathological conditions[18, 19]. We also observed that knockdown of the expression of MIF by siRNA abolished the oxLDL-mediated migration of A10 cells. The transient expression of MIF by VSMCs observed in atherogenic lesions of a hypercholesterolemic rabbit model[6] may support our present findings. In addition, the up-regulation of MIF production by VSMCs over different stages of human atherosclerosis was also reported previously[14]. These observations suggest the involvement of MIF in VSMC migration during advanced plaque formation. Of note, MIF secretion is stimulated by cytokines such as TNFα and IL-1β[5, 20], and upregulation of MIF was observed in VECs and VSMCs following inflammatory stimulation[7, 21]. These observations suggest that inflammatory response is also important for the production of MIF in atherogenesis. Furthermore, the present data support the previous findings that MIF enhances oxLDL uptake by macrophages in an autocrine and paracrine fashion. Macrophage-derived MIF may also stimulate the migration of VSMCs concomitant with the effect of VSMC-derived MIF induced by oxLDL. Thus, MIF acts as an enhancer of the progression of atherosclerosis, involving the initiating role of atherogenesis, such as fatty streak formation, and the advanced stage of atherosclerosis.

The enhanced expression of endothelial adhesion molecules, such as VCAM-1, ICAM-1, β1 and β2-integrin and P- and E-selectin, have been suggested as a key process during the progression of atherogenesis. A recent publication showed that treatment with anti-MIF antibody led to the reduction of a variety of inflammatory mediators typically associated with atherosclerosis, including circulating levels of fibrinogen and IL-6 together with the reduction of local aortic expression of ICAM-1 in apolipoprotein E-deficient mice[22]. Lin *et al.* previously showed that MIF stimulates ICAM-1 expression in endothelial cells[6]. These observations support our previous findings and present data, indicating that MIF has a potential role in atherogenesis.

Although intracellular formation of MIF/JAB1 complexes[23], and CD74-mediated triggering of the mitogen-activated protein kinase (MAPK) pathway by MIF[24-27] have been implicated, the molecular basis of the function of MIF is still incompletely understood, and the contribution of the MIF-mediated signaling pathway has not been clarified. MIF has been shown to regulate the migration of endothelial cells via activation of MAPK and phosphatidyl inositol 3 kinase (PI3K)[28]; therefore, we speculate that the effect of MIF on the migration of VSMCs may be associated with these molecules. Furthermore, the potential role of MIF in association with tumor invasion and metastasis via the Rho-dependent pathway has been reported previously[29]. In the process of tumor invasion and metastasis, migration activity is accompanied by stress fiber formation and focal adhesion assembly through a Rho/Rho-associated kinase pathway[29]. These observations indicate that the effect of MIF on the migration

of VSMCs may be associated with the Rho/Rho-associated kinase pathway.

Recently, increased plasma concentration of MIF in obese subjects has been reported[30]. These increases in MIF are related to increases in body mass index and serum levels of free fatty acids and C-reactive protein. In addition, metformin suppresses plasma MIF concentrations in the obese and this action of metformin may contribute to the antiatherogenic effect. Herder *et al*. demonstrated the association of systemic concentrations of MIF with impaired glucose tolerance and type 2 diabetes[31]. These clinical findings suggest the contribution of MIF to the development of atherosclerosis and support our hypothesis. The MIF inhibitor has been shown to inhibit the pro-inflammatory activity of MIF[32], and neutralizing anti-MIF antibody has been shown to inhibit the progression of atherosclerosis *in vivo*[15, 21]; however, these therapies have not been established in humans and further study is needed to confirm the effect of MIF blockade on the prevention of cardiovascular events.

In conclusion, MIF is involved in the initiation of the migration of VSMCs and may be associated with the development of advanced lesions during the course of atherogenesis.

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