Glycoxidation and lipid peroxidation of low-density lipoprotein can synergistically enhance atherogenesis

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Abstract

Objective: The purpose of this study was to clarify the role of glycoxidation and lipid peroxidation of low-density lipoprotein (LDL) in atherogenesis. Methods and results: We examined the formation of N-(carboxymethyl)lysine (CML), a glycoxidation product, and malondialdehyde (MDA), a lipid peroxidation product, in vitro and their co-localization in human atherosclerotic lesions. Immunohistochemical analysis revealed that CML was formed in a time-dependent manner by human LDL incubated with copper ions and glucose, i.e. an in vitro model of glycoxidation of LDL. When LDL was exposed to copper ions alone, a small amount of CML was formed, however this was significantly less in oxidized LDL than glycoxidative LDL. In contrast, MDA formation was observed in both oxidation and glycoxidation of LDL, but not in glycation of LDL. Hexitol-lysine (HL), an Amadori product, was formed by both glycation and glycoxidation of LDL, but not by oxidation of LDL. Immunohistochemical analysis showed that CML and MDA accumulated mainly in macrophage/foam cells, while pyrraline, a non-oxidative product of glycation, and apolipoprotein B were localized in the extracellular matrix in atherosclerotic lesions. Atheromas were positive for CML and MDA, but negative for pyrraline. Macrophage/foam cells in atherosclerotic lesions exhibited co-localization of macrophage scavenger receptor-A with CML and MDA, but not with pyrraline.

Conclusion: Our results suggest that glycoxidation and lipid peroxidation of LDL synergistically promote the development of atherosclerotic lesions through interaction with macrophage scavenger receptor-A. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Atherosclerosis; Diabetes; Lipoproteins

1. Introduction

A series of non-enzymatic chemical reactions between reducing sugars, proteins and lipids generate Amadori products and eventually lead to formation of advanced glycation end-products (AGEs), which comprise heterogeneous chemical compounds [1]. We have previously demonstrated that AGEs accumulate in human atherosclerotic lesions and that their location depends on different epitopes on AGEs [2]. Palinski et al. [3] demonstrated co-localization of oxidation-specific adducts and AGE-specific epitopes within intimal lesions of euglycemic WHHL rabbits. Thus, in vivo lipid peroxidation and glycation reactions may be intrinsically linked and play a causal role...
in atherogenesis. However, the respective roles of these metabolic factors in atherogenesis are not clearly established.

$N^\text{oxi}$-(carboxymethyl)lysine (CML) and pentosidine are major chemical compounds in AGEs, which form under oxidative conditions, and are referred to as glycoxidation products [4]. Previous studies have shown the presence of high levels of glycoxidation and lipid peroxidation products in serum and tissues in association with atherosclerosis, diabetes and aging [4–6]. Glycated LDL is more susceptible to oxidative modification of its lipids and protein than native LDL, suggesting the contribution of glycoxidation to oxidized LDL formation [7,8]. Amadori products and intermediates are known sources of reactive oxygen species, such as superoxide and hydrogen peroxide [9,10], which are likely to be involved in glycoxidation-induced enhancement of oxidized modification of LDL. In our previous studies [2,11,12], macrophage/foam cells in atherosclerotic lesions exhibited co-localization of CML and oxidized phosphatidylcholine, which is thought to be an early product of oxidative modification of phospholipid on LDL. However, the staining pattern of these two compounds was somewhat different in the extracellular matrix of the intima. Malondialdehyde (MDA) is known to be an end-product of lipid peroxidation formed by oxidation of LDL [13]. Moreover, MDA-modified LDL has been shown to increase in serum and tissues in association with atherogenesis [14,15]. Therefore, we examined CML and MDA formation by in vitro glycoxidation and oxidation of LDL, and their immunohistochemical localization in atherosclerotic lesions. Macrophages and macrophage-derived foam cells play an important role in atherogenesis. These cells express various scavenger receptors to bind and internalize negatively-charged macromolecules, including acetylated LDL, oxidized LDL and AGEs [16–18]. Using immunohistochemistry, Naito et al. [19] demonstrated the expression of macrophage scavenger receptor-A in macrophage/foam cells in human atherosclerotic lesions. To our knowledge, however, there are no studies that have previously demonstrated the co-localization of this receptor with glycoxidative and oxidized LDL. In this study, we demonstrate that glycoxidative LDL and oxidized LDL may synergistically promote atherosclerosis through their interaction with the macrophage scavenger receptor.

2. Methods

2.1. Chemicals

Aminoguanidine, o-phenylenediamine dihydrochloride, 3,3’ diamino-benzidine and naphthol-AS-BI-phosphoric acid were purchased from Sigma (St. Louis, MO). Ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), Na$_2$H$_4$PO$_4$, CuSO$_4$ and glucose were obtained from Wako (Osaka, Japan). Cysteine hydrochloride, bovine serum albumin (BSA), and glutathione were purchased from Seikagaku Kogyo (Tokyo, Japan). The BCA protein assay kit was purchased from Pierce (Rockford, IL). Non-immune, chromato graphically-purified rabbit and mouse IgG were obtained from Zymed (San Francisco, CA). A 96-well microtitration plate was purchased from Greiner (Frickenhausen, Germany). All other chemicals were of the best grade available from commercial sources.

2.2. Antibodies

Horseradish peroxidase (HRP)-conjugated rabbit antimouse IgG antibody was purchased from Cappel (Durham, NC). Monoclonal anti-oomy muscle actin antibody, monoclonal anti-human macrophage antibody (CD68), biotinylated rabbit anti-mouse IgG antibody, HRP-conjugated goat anti-rabbit IgG antibody and alkaline phosphatase-labeled streptavidin were obtained from Dako (Carpinteria, CA). Biotinylated goat anti-mouse IgG+IgM antibody was obtained from Chemicon (Temecula, CA). HRP-conjugated goat anti-mouse IgG+IgM antibody was obtained from Jackson ImmunoRes (West Grove, PA). Monoclonal anti-human apolipoprotein B (Apo-B) antibody was obtained from Monosan (The Netherlands). A monoclonal antibody against oxidized LDL (DLH2) was prepared as described by Itabe et al. [20]. This antibody recognizes various proteins modified by malondialdehyde (MDA), including MDA-LDL, MDA-BSA, MDA-high-density lipoprotein and MDA-hemoglobin [20]. Monoclonal anti-CML and pyrraline (PRL) antibodies were prepared by Horiiuchi et al., as previously described [21,22]. The polyclonal anti-heptoxyl-lysine (HL) and monoclonal anti-macrophage scavenger receptor-A (SRA-E5) antibodies were prepared as described by Myint et al. [23] and Takeya et al. [24], respectively.

2.3. Preparation of low-density lipoprotein

LDL was isolated from freshly drawn and pooled plasma samples (250 ml) from several consenting human donors. Native LDL (n-LDL) was prepared in the presence of 1 mM EDTA by sodium bromide stepwise density gradient ultracentrifugation. After centrifugation, fractions with density ranging from 1.019 to 1.063 were obtained as n-LDL and then dialyzed against an excess of 0.15 M NaCl and 0.25 mM EDTA (pH 7.4) at 4°C. n-LDL was sterilized by Millipore filtration (0.45 μm) and stored in the dark at 4°C. The present study conforms with the principles outlined in the Declaration of Helsinki.

2.4. In vitro oxidation, glycation and glycoxidation of low-density lipoprotein

n-LDL was dialyzed against an excess of PBS at 4°C before modification, to remove EDTA. All solutions were
sterilized by filtration through 0.45-μm pore membranes into sterile conical tubes and the modifications were carried out at 37°C for 1 or 2 weeks under sterile conditions. Oxidized LDL (ox-LDL) was prepared by incubating n-LDL with 5 μM CuSO₄ in 0.2 M phosphate buffer (pH 7.4). Glycated LDL (gl-LDL) was prepared by incubation of n-LDL with 250 mM glucose in the presence of 1 mM EDTA and 1 mM DTPA. Glycoxidative modification of LDL (go-LDL) was performed by incubating n-LDL with both 5 μM CuSO₄ and 250 mM glucose in 0.2 M phosphate buffer (pH 7.4). Non-glycated/non-oxidized LDL (ngo-LDL) was prepared by incubating n-LDL in 0.2 M phosphate buffer (pH 7.4) containing 1 mM EDTA/1 mM DTPA. The modification was stopped by extensive dialysis of the reaction mixtures against 0.15 M NaCl and 0.25 mM EDTA at 4°C. Protein concentration was determined using the BCA protein assay kit.

2.5. Quantification of CML, HL and MDA formation by non-competitive ELISA

The formation of CML, HL and MDA was estimated using non-competitive ELISA. All procedures were carried out at room temperature. The wells of microtiter plates were coated with various concentrations of samples in 0.1 ml carbonate buffer for 60 min. For HL assay, samples were diluted with PBS (pH 7.4) and simultaneously reduced by adding 0.1 M NaBH₄ solution [23]. Wells were washed three times with wash buffer (PBS containing 0.05% Tween 20), and then incubated with 0.5% gelatin for 60 min to block non-specific binding. After washing again with wash buffer, 50 μl of anti-CML, anti-MDA or anti-HL antibody in PBS containing 0.1% BSA and 0.05% Tween 20 was added to each well and incubated for 60 min. After further washings, the wells were incubated with 50 μl peroxidase-conjugated secondary antibody for 60 min. The reactivity of peroxidase was determined by incubation with o-phenylenediamine dihydrochloride for appropriate intervals and absorbance at 492 nm measured on an ELISA plate reader. Glycated BSA [23], AGE-BSA [21] and MDA-LDL [20] were used as standards for HL, CML and MDA assays, respectively.

2.6. Effects of chelators and aminoguanidine on formation of CML, HL and MDA

Incubation of n-LDL with copper ions and glucose was carried out at 37°C for 2 weeks in the presence or absence of metal chelators, including EDTA (1 mM) and DTPA (1 mM), and aminoguanidine (100 mM). After extensive dialysis against PBS containing 0.25 mM EDTA, we determined the formation of CML, HL and MDA using non-competitive ELISA, as described. The inhibitory effect of chelators and aminoguanidine was expressed as the percentage decrease in absorbance at 492 nm (% inhibition): % inhibition = 100 × [(ABSa − ABSb)/(ABSc − ABSb)], where ABSa and ABSc indicate absorbances of glycoxidative LDL with and without inhibitors, respectively. ABSb indicates those of n-LDL.

2.7. Arterial samples

Sections of arterial walls were obtained from ten males and ten females at autopsy within 6 h of death (mean age, 58.5±13.9 years; range, 31–84 years). Table 1 lists the cause of death in each subject. All subjects were both clinically and pathologically free of diabetes mellitus and chronic renal failure. As shown in Table 1, samples for immunohistochemical analysis comprised 28 grossly normal regions (diffuse intimal thickening, DIT), 24 fatty streaks (FS) and 23 atherosclerotic plaques (AP). Complicated lesions with calcification and ulceration were excluded from the study. Tissue specimens were washed with PBS and then fixed with Carnoy’s fixative for 2 h. After dehydration with ethanol, tissues were embedded in paraffin. Paraffin sections were prepared and stained with hematoxylin and eosin.

2.8. Immunohistochemical methods

Immunohistochemical localization of various antigens, including CML, MDA, PRL and Apo-B, was examined using the labeled streptavidin–biotin technique. Briefly, serial tissue sections were deparaffinized and then subjected to protease pretreatment at room temperature for 10 min for CML and PRL. After treatment with 3% BSA for 30 min, the slides were reacted with primary antibody at 4°C overnight. The primary antibodies used in this study included monoclonal anti-CML (6D12), anti-PRL (H12) anti-MDA (DLH2) and anti-Apo-B antibodies, as well as monoclonal anti-α-smooth muscle actin and anti-human macrophage (CD68) antibodies. After additional washes (in PBS, three times), biotinylated anti-mouse IgG antibody (1:200) was added for 30 min at room temperature. The slides were then rinsed with PBS and incubated for 30 min with alkaline phosphatase-labeled streptavidin (1:100) at room temperature. The alkaline phosphatase coloring reaction was carried out by incubating the slides with naphthol-AS-BI-phosphoric acid as a substrate and hexazonitized new fuchsin as a coupler in 0.2 M Tris–HCl buffer (pH 8.2). The reaction mixture contained levamisole (24 mg/ml) to block endogenous alkaline phosphatase activity. After washing with PBS, sections were counterstained with Meyer hematoxylin. Control tests for specificity of immunostaining included substitution of non-immune sera or PBS for primary antibodies and competitive test with antibodies preincubated with excess of CML-bovine serum albumin, MDA-LDL or synthesized pyrraline. To evaluate the interaction between macrophages and modified LDL, co-localization of macrophage scavenger receptor-A with CML, MDA and PRL in atherosclerotic lesions was assessed using an immunohistochemical double-staining.
Table 1
Clinical data and number of examined atherosclerotic lesions in 20 autopsies

<table>
<thead>
<tr>
<th>No.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Cause of death</th>
<th>Number of lesions examined</th>
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<tr>
<td>2</td>
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<td>M</td>
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<td>43</td>
<td>M</td>
<td>Glioblastoma</td>
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<tr>
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<td>43</td>
<td>F</td>
<td>Aspiration pneumonia</td>
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<td>F</td>
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<td>F</td>
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<td>M</td>
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<td>M</td>
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<td>M</td>
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<td>F</td>
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<tr>
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<td>84</td>
<td>F</td>
<td>Lung carcinoma</td>
<td>0  1  1</td>
</tr>
</tbody>
</table>

Total number of samples 28 24 23

*AP, atheromatous plaque; DIT, diffuse intimal thickening; FS, fatty streak.

Technology. In the first step, deparaffinized sections were stained with monoclonal anti-macrophage scavenger receptor-A antibody (SRA-E5). After visualization of peroxidase activity localization with 3,3′-diaminobenzidine as a substrate, sections were rinsed with 0.1 M glycine–HCl buffer (pH 2.2) for 60 min. In the second step, the same sections were incubated with anti-CML, anti-MDA or anti-PRL antibody. The alkaline phosphatase coloring reaction was performed as described before.

2.9. Statistical analysis

Numerical data were expressed as mean±S.D. In vitro data showed a normal distribution. In conclusion, differences between groups were examined for statistical significance by using Student’s unpaired t-test. P-values of less than 0.05 were considered significant.

3. Results

3.1. Formation of CML, MDA and HL in low-density lipoprotein by glycation, oxidation and glycoxidation

Fig. 1 shows the formation of CML, MDA and HL in various modified LDLs. The formation of CML is shown in Fig. 1A. Slow but significant time-dependent formation of CML was observed when LDL was incubated with both glucose and copper ions, indicating that glycoxidation of LDL may result in CML formation. In contrast, LDL incubated with either copper ions or glucose for 1 week (oxidized or glycated LDL) showed no increase in immunoreactivity to anti-CML antibody. LDL incubated with copper ions for 2 weeks formed CML to some extent, however the amount was significantly smaller than glycoxidative LDL. As shown in Fig. 1B, LDLs incubated with copper ions or copper ions and glucose, i.e. oxidized and glycoxidative LDLs, significantly increased the immunoreactivity to anti-MDA antibody after 1 and 2 weeks. No difference in the intensity of immunoreactivity to anti-MDA antibody was observed between oxidized and glycoxidative LDLs. In contrast, glycation of LDL did not lead to MDA formation. Fig. 1C shows HL formation in various modified LDLs. Significant formation of HL was observed in both glycated and glycoxidative LDLs after 1 and 2 weeks. The intensity of immunoreactivity of glycated LDL with anti-HL antibody was similar to that of glycoxidative LDL after 1 and 2 weeks. In contrast, oxidized LDL exhibited no HL formation.

3.2. Effects of chelators and aminoguanidine on the formation of CML, HL and MDA

Next, we examined the effects of chelators and aminoguanidine on the formation of CML, HL and MDA.
Fig. 1. Immunochemical detection of \(N^\prime\)-(carboxymethyl)lysine (A), malondialdehyde (B) and hexitol-lysine (C) in oxidized, glycoxidative, glycated and non-oxidized/non-glycated LDLs. Oxidized (a), glycoxidative (b) and glycated (c) LDLs were prepared by incubating native LDL (e) with copper ions, copper ions plus glucose and glucose plus EDTA/DTPA in phosphate buffer at 37°C for 1 or 2 weeks, respectively. Non-oxidized/non-glycated LDL (d) was prepared by incubating n-LDL in phosphate buffer containing EDTA/DTPA at 37°C. Then 1-μg/ml of samples were coated as antigen onto microtiter wells. The CML, MDA and HL in samples were determined by non-competitive ELISA, as described in Methods. Statistical analysis was performed using Student’s unpaired \(t\)-test. (A) \(P<0.0005\), versus oxidized, glycated and non-oxidized/non-glycated LDLs; \(P<0.0001\), versus oxidized, glycated and non-oxidized/non-glycated LDLs, \(P<0.0005\), versus glycoxidative LDL of 1 week; \(P<0.005\), versus glycated and non-oxidized/non-glycated LDLs. (B) \(P<0.0001\), versus glycated and non-oxidized/non-glycated LDLs. (C) \(P<0.0001\), versus oxidized and non-oxidized/non-glycated LDLs.

Fig. 2 shows the immunohistochemical localization of CML and pyrraline (PRL) in diffuse intimal thickening (DIT). CML was found in a few smooth muscle cells and extracellular matrix in about one third of DIT. Similarly, MDA was observed mainly in intimal smooth muscle cells. In contrast, PRL and Apo-B were found exclusively in extracellular matrix in over half the DIT. In fatty streaks (FS), the majority of foam cells exhibited positive staining for CML and MDA (Fig. 3A and B). Staining of serial sections with antibody against macrophage marker CD68 confirmed that the foam cells were macrophage-derived (Fig. 3E). A proportion of smooth muscle cells was also positive for CML and MDA. In contrast, PRL and Apo-B were found exclusively in extracellular matrix in FS (Fig.
Fig. 3. Comparative immunolocalization of malondialdehyde, \(N^\prime\)-(carboxymethyl) lysine, pyrraline and apolipoprotein B in fatty streaks. The tissue sample was obtained from the aorta of a 59-year-old male who died due to colonic carcinoma. Serial sections were stained with monoclonal anti-MDA (A), anti-CML (B), anti-PRL (C) and anti-Apo-B (D) antibodies. Macrophages and smooth muscle cells were identified by immunostaining with CD68 (E) and smooth muscle actin (F), respectively. Note that the majority of macrophage/foam cells within the lesion were positive for MDA and CML (A, B, open arrows). Note also that some smooth muscle cells are positive for MDA and CML (A, B, hatched arrows). In contrast, PRL and Apo-B are mainly in extracellular matrix within the lesion (C, D, arrows). Original magnification is \(\times270\) for all photomicrographs.

3C and D). Fig. 4 shows representative immunohistochemical localization of various antigens in atheromatous plaques (AP). CML and MDA were observed in both macrophage/foam cells and smooth muscle cells, and also in extracellular matrix and atheromas (Fig. 4A, B, D and E). In contrast, PRL and Apo-B were sparse in macrophage/foam cells and smooth muscle cells, but were detected in the extracellular matrix of AP (Fig. 4C, F and G). CML and MDA, but not PRL, were found in atheromas in most AP (Fig. 4A, B and C).

3.4. Co-localization of macrophage scavenger receptors with CML, MDA and PRL

We used the double-staining method to illustrate the interaction between macrophage scavenger receptors and glycoxidative and oxidized LDL. As shown in Fig. 5D and E, most of the CD68-positive foam cells in atherosclerotic lesions expressed scavenger receptor-A. The cytoplasm of these foam cells was also positive for CML and MDA (Fig. 5A and B). MDA was also detected in smooth muscle cells, which were negative for CD68 and SRA-E5. In contrast, PRL was detected in the extracellular matrix, but not in foam cells expressing macrophage scavenger receptor-A (Fig. 5C).

4. Discussion

Non-enzymatic glycation between reducing sugars and proteins produces Amadori compounds and, subsequently, leads to formation of AGEs. Recent studies have shown that AGEs are generated via both oxidative and non-oxidative pathways. CML is an oxidative AGE, i.e. a
Fig. 4. Comparative immunolocalization of malondialdehyde, \(N\)-[(carboxymethyl)lysine, pyrraline and apolipoprotein B in atherosclerotic plaque. The tissue sample was obtained from the aorta of a 70-year-old female who died due to malignant lymphoma. Serial sections were stained with monoclonal anti-MDA (A, D), anti-CML (B, E), anti-PRL (C, F) and Apo-B (G) antibodies. Macrophages and smooth muscle cells were identified by immunostaining with CD68 (H) and smooth muscle actin (I), respectively. Note that the majority of macrophage/foam cells and smooth muscle cells within the lesion are positive for MDA (D). CML is present in parts of these two cells (E). In contrast, PRL and Apo-B are mainly found in extracellular matrix (C, *; F, G, hatched arrows) within the lesion. Note also that the atheroma is positive for MDA and CML, but negative for PRL (A, B, C, star). Original magnification of photomicrographs is \(\times 135\) for A–C and \(\times 270\) for D–I.
Fig. 5. Co-localization of macrophage scavenger receptor-A with malondialdehyde, N’-(carboxymethyl)lysine and pyrraline in atheromatous plaque. The tissue sample was obtained from the aorta of a 70-year-old male who died due to prostatic carcinoma. Double-staining of macrophage scavenger receptor-A with MDA (A), CML (B) and PRL (C) was performed as described in Methods. In serial sections, macrophage scavenger receptor-A and macrophages were identified by immunostaining with SRA-E5 (D) and CD68 (E), respectively. Note that the majority of macrophage/foam cells within the lesion express macrophage scavenger receptor-A (D and E). In double-stained sections, most macrophage/foam cells (A, B, arrows) show co-localization of macrophage scavenger receptor-A (brown) and MDA (red) or macrophage scavenger receptor-A (brown) and CML (red). In contrast, PRL (C, red) is predominantly deposited in extracellular matrix in the subluminal region of intima, but not co-localized with macrophage scavenger receptor-A expressed in macrophage/foam cells (brown). Original magnification is ×400 for all photomicrographs.
glycoxidation may cooperate with lipid peroxidation of LDL during development of atherosclerotic lesions.

Our study provided, for the first time, indirect evidence for co-localization of macrophage scavenger receptor-A with glycoxidative and oxidized LDLs in macrophage/foam cells in human atherosclerotic lesions. Macrophage scavenger receptor-A is one of the major scavenger receptors of macrophages and has been identified in various tissues, including lung, liver, brain, kidney and aorta [18,19,24]. This receptor is known to bind and internalize various negatively-charged macromolecules, including acetylated LDL, oxidized LDL and AGEs, and to contribute to foam cell development in atherogenesis [18]. However, to our knowledge, there are no studies that have previously shown co-localization of macrophage scavenger receptor-A with oxidized LDL and AGE-modified LDL in macrophage/foam cells in atherosclerotic lesions. Using double-immunostaining analysis, we demonstrated here the expression of macrophage scavenger receptor-A in macrophage/foam cells in atherosclerotic lesions and simultaneous accumulation of CML and MDA in the cytoplasm of these cells. In contrast, PRL was deposited in extracellular spaces in intima, but did not co-localize with macrophage scavenger receptor-A. CML and MDA were formed by in vitro glycoxidation and oxidation of LDL, respectively. Thus, glycoxidative and oxidized LDLs may synergistically enhance foam cell development via their interaction with macrophage scavenger receptor-A.

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