The Development of ELISA Methods for the Measurement of Oxidized LDLs and Autoantibodies Against Oxidized LDLs in Human Serum

Katherine Ann Garvey
College of Saint Benedict/Saint John's University

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The Development of ELISA Methods for the Measurement of Oxidized LDLs and Autoantibodies Against Oxidized LDLs in Human Serum

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By

Katherine Ann Garvey

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Abstract

A significant risk factor for the development of heart disease appears to be oxidized low-density lipoproteins (oxLDLs). Currently there are no methods which specifically measure oxidized LDLs in serum. ELISA methods were developed which specifically detect oxLDLs and autoantibodies against oxLDLs in human serum. These sensitive assays can detect oxLDLs in concentrations as small as 50 ng/mL serum. Optimal assay conditions such as serum and antibody dilutions, as well as blocking agents and washing buffers were determined. The assays have successfully detected greater amounts of oxLDLs and autoantibodies in more concentrated serum dilutions. Ultimately these assays may be used to quantify levels of oxidatively modified LDLs in patient serum. If oxLDLs could be correlated with other risk factors, it may be possible to more accurately predict a patient's risk for developing heart disease.

Introduction

Heart disease is the leading cause of death in Western populations (1). There are a number of identified factors which increase an individual's risk for developing heart disease including: elevated levels of total cholesterol (hypercholesterolemia) and low density lipoproteins (LDLs) (1-4), decreased levels of high density lipoproteins (HDLs) (3), hypertension and smoking (5). However, these factors only account for half of all cases of heart disease (6). In the search for additional risk factors, it was recognized that LDLs can become oxidized, and these oxidatively modified LDLs appear to be far more atherogenic than unmodified LDLs. Levels of oxidized LDLs (oxLDLs) (7) or autoantibodies against oxLDLs (8-11) are higher in patients with atherosclerosis than in individuals without heart disease. Hence people with normal LDL levels may be at increased risk, but would be missed by screening only total LDL levels. Measuring the concentration of oxLDLs and autoantibodies against oxLDLs may alert individuals who do not display the classic risk factors for heart disease. Therefore, levels of oxLDLs may be a better predictor of risk than levels of unmodified LDLs (12).

OxLDLs are taken up by unregulated scavenger receptors on macrophages at a rate three to ten times faster than native LDLs which are taken up through a feedback-controlled process
The accelerated rate of oxLDLs uptake leads to cholesterol accumulation and foam cell formation, which are the initial events in the development of an atherosclerotic lesion (3). Currently there is no method to directly measure the amount of oxLDLs present in serum, so individuals with this particular risk go unrecognized. The development of a method which can specifically measure oxLDLs and correlate oxLDLs with other risk factors will allow for a more accurate prediction of a patient’s risk for developing heart disease.

**Literature Review**

*Oxidation in vivo*

LDLs are oxidized through a series of steps, such as described below (Figure 1). A radical species (a highly reactive molecule which has an unpaired electron) abstracts a hydrogen from a double bond in a polyunsaturated fatty acid (PUFA) on the surface of the LDL. This forms a fatty acid radical which can now abstract hydrogens from neighboring PUFA, continuing the oxidation process (12). The removal of a hydrogen from the fatty acid is followed by molecular rearrangement of the double bonds into a more stable conjugated diene form (13). Antioxidants such as vitamin E, which is transported within LDLs (14), can interrupt this sequence by reacting with the radical species. However, once antioxidants are depleted, the oxidation of PUFA begins again (15) and oxidation can spread into the lipid core of the LDL particle. When PUFA break down, aldehydes (such as malondialdehyde) and other metabolites are produced which may react with lysine residues on apolipoprotein B, generating an oxidized LDL molecule which is recognized by macrophage scavenger receptors (12).
Figure 1. Oxidation Mechanism (Adapted from ref. 1)

1) Hydroxy radical abstracts a hydrogen from a PUFA
2) Molecular Rearrangement into a more conjugated diene form
3) O₂ attacks PUFA
4) Lipid peroxyl radical abstracts a hydrogen from another PUFA

If high concentrations of antioxidants are present in the plasma, it is unlikely that LDL oxidation occurs in the circulation (2, 12, 16-19). Under these conditions LDL oxidation would only occur in sequestered microenvironments of the intima, away from the protection of antioxidants (12, 20). Once oxidized, LDLs could leak back into the circulation (20, 21) and trigger antibody production. The finding that circulating oxLDLs and oxLDLs isolated from atherosclerotic lesions have the same characteristics supports the possibility that oxLDLs can leak back into the circulation (21). It is also possible that oxidation may occur in a secluded portion of the LDLs while in the circulation, especially when antioxidant levels are low (20).

The partial oxidation of LDLs in the subendothelial space triggers a series of events leading to the development of an atherosclerotic lesion. Minimally-modified LDLs (MM-LDLs) may induce the secretion of monocyte chemotactic protein-1 (MCP-1) and the expression of
adhesion molecules for monocytes on the endothelium. This results in monocyte attachment to the endothelium and movement of monocytes into the subendothelial space (2, 22). MM-LDLs may also induce the secretion of macrophage colony stimulating factor (M-CSF) which facilitates the differentiation of monocytes into macrophages. Macrophages can further oxidize the MM-LDLs into fully oxidized LDLs that are taken up by scavenger receptors (2, 22). In addition to facilitating the movement of monocytes into the subendothelial space, oxLDLs also retard the movement of macrophages out of the subendothelium, leading to macrophage accumulation and the development of a fatty streak (2).

In addition to its role in the formation of foam cells, oxLDLs have other atherogenic characteristics as well. OxLDLs are cytotoxic to cells in the arterial wall, and may damage endothelial cells, allowing LDLs and monocytes to more easily move into the subendothelial space where lesion formation takes place (2). OxLDLs also inhibit vasodilation, enhance coagulation, and increase platelet activation (23), all of which may contribute to lesion formation. Another characteristic of oxLDLs is their immunogenicity, meaning they trigger an immune response that results in the production of IgG autoantibodies against oxLDLs (2, 24).

Current Methods

Two current methods for estimating oxLDLs are monitoring conjugated diene formation and the measurement of thiobarbituric acid reactive substances (TBARS), such as malondialdehyde, which are formed during the oxidation of LDLs. Conjugated dienes are produced during the oxidation of polyunsaturated fatty acids (PUFA) contained in the LDLs when the double bonds of the PUFA rearrange into a more stable conjugated form (See Figure 1) (13). Because conjugated dienes absorb ultraviolet light at 234 nm, their formation can be monitored as an increase in absorbance at 234 nm (13).
While conjugated diene formation is often used as a means to estimate susceptibility of LDLs to oxidation in vitro (8,13,25-27), this method does not exclusively detect conjugated dienes formed due to LDL oxidation. In addition to oxLDLs, all substances which absorb UV light at 234 nm are detected (28), so that the assay may actually overestimate the presence of oxLDLs. While this method can measure how resistant LDLs are to oxidation, it cannot determine the extent to which LDLs were previously oxidized in vivo. If a subject’s LDLs appear to be highly resistant to oxidation, this method does not differentiate between resistance due to a high concentration of antioxidants, a low concentration of polyunsaturated fatty acids, or a high concentration of LDLs that have already been extensively oxidized in vivo.

The TBARS assay determines the amount of malondialdehyde (MDA) generated during oxidation (26). MDA is produced during the catabolism of arachidonic acid by platelets at sites of arterial injury and during the decomposition of unsaturated fatty acids by monocytes during phagocytosis (29). One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA), and the formation of MDA-TBA adducts is measured at 562 nm (1). However, some sugars and amino acids can also form adducts with TBA, thus making this assay nonspecific as well (4). In addition, the presence of aldehydes is not exclusively due to LDL oxidation, since aldehydes may potentially be produced during the metabolism of unsaturated fatty acids (26). Furthermore, the harsh conditions of the assay itself initiate oxidation beyond that which occurred in vivo (1,13,30); for example, heating samples at 95°C during the assay increases the formation of TBARS nearly ten-fold (30) and generates artificially high measurements of oxidation products.

The presence of autoantibodies against oxLDLs (8-10, 24, 31-34) and the fact that oxLDLs can be detected in serum, suggests that oxLDLs may circulate in the blood. If oxLDLs
can be detected and quantified, this data could be utilized in conjunction with the classic risk factors as a more precise means of assessing a patient's risk of developing atherosclerosis.

The development of enzyme-linked immunosorbent assay (ELISA) methods can provide a means for directly measuring oxLDLs and autoantibodies against oxLDLs in serum. Extreme temperatures or acidic solutions are not required, so the possibility of further oxidation of samples is low. Figure 2 shows a basic ELISA procedure. The wells of a microtiter plate are coated with antigen (oxLDLs) overnight. After unbound antigen is removed from the wells, a blocking agent is added which binds remaining open sites on the plate to prevent nonspecific protein binding later in the procedure. After excess blocking agent is removed, serum samples are added to the wells, along with the first antibody, which is specific for oxLDLs. The antibody binds to oxLDLs present in the serum, and remaining antibody passes through the serum and binds to the oxLDLs coated on the bottom of the plate. After two hours of incubation, the serum and antibody are removed and a second antibody is added, which binds to the first antibody. The second antibody has an enzyme linked to it so the addition of a substrate produces a color that can be measured spectrophotometrically and quantified.
Figure 2. ELISA Procedure

1. Antigen (oxLDL) bound to plate
2. Unbound antigen is removed, blocking agent is added.
3. Unbound blocking agent is removed, serum and first antibody are added.
4. Unbound serum and first antibody are removed, enzyme-linked second antibody is added.
5. Unbound second antibody is removed, substrate is added.
Methods

Preparation of antigen for ELISA

LDLs (Calbiochem) were oxidized using malondialdehyde following the procedure of Haberland (29). 100 μL LDLs (10 mg/mL) and 100 μL malondialdehyde were transferred to a bullet tube and incubated at 37°C for three hours. After incubation, the oLDLs were collected from the bullet tube and injected into a microdialyzer cassette (Pierce) and dialyzed against 200 mL of dialysis buffer (4.383 g NaCl, 0.05 g disodium EDTA, 50 mL sodium phosphate buffer, and H₂O to a final volume of 500 mL) in a covered beaker at 4°C for 16 hours to remove unbound malondialdehyde. See Appendix for detailed dialysis procedure.

Western Blotting

Gel Electrophoresis compared the mobility of the oLDLs to that of native LDLs on agarose gel (see Appendix for detailed gel electrophoresis procedure), and Western Blotting confirmed the specificity of the antibodies for oLDLs. Samples of native and oxidized LDLs were run through agarose gel. Gershoni’s Buffer Solution (1.89 g Tris, 9 g glycine, and 1 L H₂O) was prepared according to Dunbar (35) and kept refrigerated until use during Western Blotting. Fiber pads, nitrocellulose paper, and filter paper were soaked in cold Gershoni’s Buffer and layered in the gel holder cassette with the agarose gel following gel electrophoresis. Cold Gershoni’s Buffer was poured into the Western Blot chamber and the power source was set at 256 mA. After one hour the nitrocellulose was removed from the cassette. At this point the LDL samples on the agarose gel were irreversibly transferred from the agarose to the nitrocellulose. The nitrocellulose was soaked in 40 mL Tween blocking buffer (5.809 g NaCl, 13 mL 2 M Tris, 500 μL Tween-20, and 1 L H₂O) (35) for one hour to bind any remaining open sites on the nitrocellulose to minimize non-specific protein binding later in the procedure. The nitrocellulose was incubated
in primary antibody (mouse anti-human oxLDL, Biodesign, diluted 1:1000 in blocking buffer). After one hour the nitrocellulose was washed in Tween blocking buffer three times (10 minutes per wash) to remove excess antibody, and incubated with secondary antibody (alkaline phosphatase-conjugated goat anti-mouse IgG, Sigma, diluted 1:1000 in blocking buffer). The excess secondary antibody was removed after one hour. In the final step the nitrocellulose was incubated in a freshly prepared substrate solution (31 mg 4-chloro-1-naphthol dissolved in 10 mL Methanol, 50 mL TBS, and 50µL of 30% hydrogen peroxide) to develop colored bands.

* The microtiter plate was rocked on a Vari-Mix (Barnstead/Thermolyne) at room temperature for all incubation steps with antibodies or blocking agents during Western Blotting and ELISA procedures.

ELISA Procedure

Malondialdehyde-oxidized LDLs (oxLDLs) were diluted in Phosphate Buffered Saline (PBS) (30µL of 5 mg/mL oxLDL in 14.97 mL PBS) for a final concentration of 0.01 mg/mL. A volume of 150 µL of 0.01 mg/mL oxLDLs was added to each well of a 96-well flat-bottom E.I.A-R.I.A microtiter plate (Bio-Rad). The plate was covered with saran wrap and incubated at 4°C for 21-22 hours. Unbound oxLDLs were removed from the wells (via shaking and inversion of plate), and 300µL of 5% BSA-PBS (Bovine Serum Albumin in PBS) was added to block sites on the microtiter plate not bound with oxLDLs. After 1-2 hours of blocking, excess blocking agent was removed and the wells were washed three times with 1% BSA-PBS, followed by addition of 150 µL serum samples diluted in 1% BSA-PBS.

If the intent of the ELISA was to detect oxLDLs in the serum, 150 µL of primary antibody (mouse anti-human oxLDL, Biodesign, diluted 1:2000 in PBS) was added immediately following addition of the serum samples. Samples were incubated for two hours followed by removal of
excess sample and three washings with 1% BSA-PBS. A volume of 150 μL of secondary antibody (alkaline phosphatase-conjugated goat anti-mouse IgG, Sigma, diluted 1:5000 in TBS-skim milk) was added to the wells and incubated for two hours. Unbound secondary antibody was removed and wells were washed three times with 1% BSA-PBS. To prepare the substrate, 2.213 g 3-[Cyclohexylamino] - 1 - propanesulfonic acid (CAPS) was added to 10 mL H₂O. The pH was adjusted to 9.8 with NaOH to facilitate dissolving of CAPS into solution. Immediately prior to development, p-nitrophenylphosphate disodium salt hexahydrate was added to the CAPS solution for a final concentration of 1 mg/mL, and 0.1 M MgCl₂ was added for a final concentration of 0.02% MgCl₂. A volume of 105 μL of substrate was added (via octapipet) to the wells and the absorbance was read every ten minutes on a Vmax kinetic microplate reader (Molecular Devices) at 405 nm against a substrate blank.

When the ELISA was intended to detect autoantibodies against oxLDLs in the serum, the antigen coating and blocking steps were the same as described above. Serum samples were added to the wells as above, but a primary antibody was not added since autoantibodies are already present in the serum. The free autoantibodies in the serum bind to the oxLDLs coated on the bottom of the plate. After removal of excess serum samples and washing, 150μL of a secondary antibody specific for human IgG (alkaline phosphatase-conjugated goat anti-human IgG, Sigma, diluted 1:10,000 in TBS-skim milk) was added to the wells. After excess samples were removed and wells were washed, the substrate was prepared and added as described above.
Other Procedures

IgG-free serum was collected using a Protein A Sepharose (Sigma) column following manufacturer instructions (see Appendix for detailed procedure).

Cu^{2+}-facilitated LDL oxidation was conducted and conjugated diene formation was monitored at 234 nm. (See Appendix for detailed procedure).
Results

Electrophoresis of oxLDLs and native LDLs showed that malondialdehyde-oxidized LDLs traveled further through agarose gel than native LDLs. Western Blotting verified that the mouse anti-human oxLDL antibody specifically bound to oxLDLs and not to native LDLs. While the mouse anti-human oxLDL antibody bound to malondialdehyde-oxidized LDLs, it did not bind to Cu^{2+}-oxidized LDLs. This demonstrates that the antibody is very specific for the change that occurs on the LDLs when it is modified with MDA but not when LDLs are oxidized with copper ions. The increase in absorbance at 234 nm (Graph 1) indicates the production of oxidized LDLs. Conjugated dienes, which are produced during the oxidation of PUFAs contained in the LDLs, absorb light at 234 nm (13).

Graph 1. CuSO₄ Oxidation
Several concentrations of oxLDLs were compared for the coating step of the ELISA; ultimately 0.01 mg/mL was chosen as the standard coating concentration (Graph 2). More concentrated oxLDL (0.10 mg/mL) yielded nearly the same absorbance readings as this lower level. Therefore, to maximize cost effectiveness, 0.01 mg/mL oxLDLs was chosen as the standard concentration.

**Graph 2. Comparison of oxLDLs concentrations for Coating Plate**

![Graph showing absorbance over time for different oxLDL concentrations](image)

*Measuring OxLDL in serum*

With this ELISA procedure, the higher the absorbance values, the lower the levels of oxLDLs present in the serum, and vice versa. When primary antibody specific for oxLDLs is added to the sample it binds to oxLDLs in the serum. Consequently, the higher the oxLDLs
present in the serum, the less remaining primary antibody available (unbound) to pass through the serum and bind to the oxLDLs coated on the plate. The secondary antibody specifically binds to the primary antibody which is now bound to the oxLDLs on the bottom of the plate. The secondary antibody has an enzyme linked to it which produces color when substrate is added. The more color production, the higher the absorbance reading. More color production demonstrates that more enzyme is present with secondary antibody. When more antibody is bound, this indicates that less oxLDLs were present in the serum to complex with primary antibody. Therefore, higher absorbance readings correlate with less oxLDLs and lower absorbance readings correlate with more oxLDLs in the serum.

Different concentrations of antibody were compared to determine the standard dilutions to be used during the ELISA procedures (see Graph 3). Dilutions of 1:2000 for the primary antibody (mouse anti-human oxLDL) and 1:5000 for the secondary antibody (alkaline phosphatase-conjugated goat anti-mouse IgG) were chosen because of the lack of absorbance generated by more dilute antibody. Specificity of the primary antibody for oxLDL was confirmed (see Graph 4); the antibody to oxLDLs did not bind to wells coated with BSA or LDLs, only oxLDLs.
Graph 3. Comparison of Antibody Dilutions

Graph 4. Specificity of Antibody for oxLDL
The initial ELISA procedure was based on suggestions by Sigma in which the wells were washed immediately after antigen binding and then blocked. Theoretically, the most concentrated serum should contain the most oxLDLs and give the lowest absorbance readings, and the least concentrated serum should give the highest absorbance readings; however, initial results did not follow this trend (see Graph 5).

**Graph 5. OxLDLs in Serum Dilutions. Early Results.**

![Graph showing absorbance levels over time for different serum dilutions](image)

It is likely that non-specific protein binding occurred during the original ELISA protocol. When the order of the washing and blocking steps was reversed, this allowed for more effective blocking of open sites on the plate. With the revised procedure, absorbance values indicate that undiluted serum contains a higher concentration of oxLDLs compared to diluted serum samples (see Graph 6).
The sensitivity of the assay to detect differences between ten-fold dilutions of oxLDLs added to the serum is evident in Graph 7. The assay can detect concentrations as small as 50 ng/mL (oxLDL diluted 1:100,000) in serum. It is imperative that the assay is sensitive so that small levels of oxLDLs can be assessed and correlated with risk.

Graph 6. OxLDLs in Different Serum Dilutions
Graph 7. Various concentrations of oxLDLs added to serum

Autoantibodies against oxLDL in serum

The ELISA procedure can also detect autoantibodies against oxLDLs in serum. Autoantibodies present in the serum bind to the oxLDLs on the bottom of the plate. More concentrated serum will contain more autoantibodies, therefore more binding to the plate will occur. With more autoantibodies attached to the plate, more enzyme-linked secondary antibody can bind and a higher absorbance value will be produced.

The antibody dilution chosen for the secondary antibody (specific for human IgG) was determined to be 1:10,000. Again, initial results did not indicate that undiluted serum contained
the highest amount of autoantibodies (see Graph 8), but when the first washing (1% BSA-PBS) and blocking (5% BSA-PBS) steps were reversed, data indicated that more concentrated serum contains more autoantibodies than less concentrated serum (Graph 9).

Graph 8. Detection of Autoantibodies in Different Serum Dilutions. Early Results

![Graph showing absorbance at 405 nm over time for different serum dilutions.](image-url)
The specificity of the secondary antibody (mouse anti-human IgG) for human IgG was confirmed (Graph 9). Absorbance readings obtained when coating the bottom of the microtiter plate with LDLs or BSA instead of oxLDLs suggest that humans produce autoantibodies against LDLs and BSA as well (see Graph 10). High levels of IgG antibodies against BSA have been detected in human serum (36). While it is likely that human serum contains a higher concentration of native LDLs than oxLDLs, it is unclear whether human serum would contain a higher level of BSA than oxLDLs. Perhaps if individuals consume beef products on a regular
basis, it is possible that some BSA is absorbed and that its presence generates an immune response.

Graph 10. Autoantibodies in Serum. Different Plate Coatings
The serum was spiked with various concentrations of oxLDLs to determine the effect this had on autoantibody binding to the oxLDLs on the plate. More oxLDLs added to the serum should facilitate more complexing with autoantibodies, resulting in less free autoantibodies to bind on the bottom of the plate. However, adding larger volumes of oxLDLs to the serum actually resulted in higher absorbance readings. This suggests that some of the added oxLDLs were binding to the plate and increased the plate coating and lead to increased antibody binding.

Graph 11. Detection of Autoantibodies in Undiluted Serum with Increasing Volumes of oxLDLs added.
The blocking capabilities of 5% BSA-PBS and TBS-skim milk were compared, as shown in Graph 12. The lower absorbance readings suggest that less nonspecific binding occurs when BSA-PBS is used, demonstrating that it is a better blocking agent than TBS-skim milk. To verify that the BSA-PBS was actually decreasing the incidence of non-specific binding, an ELISA was done in which some wells were not blocked after coating the plate with oxLDLs (see Graph 13).

Graph 12. Comparison of BSA-PBS and TBS-skim Blocking Agents
Graph 13. Comparison of Blocked Wells and Non-Blocked Wells

Absorbance @ 405 nm

Time (min)

1:10 serum, No Blocking
1:10 serum, BSA-PBS Blocking
## Summary of ELISA Results

### ELISA for oxLDLs

<table>
<thead>
<tr>
<th>Theoretical</th>
<th>Experimental</th>
<th>Pages in Notebook</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No interaction with BSA coat</td>
<td>No interaction</td>
<td>Notebook II pg 48</td>
</tr>
<tr>
<td>2. No interaction with LDLs coat</td>
<td>No interaction</td>
<td>II pg 48</td>
</tr>
<tr>
<td>3. ↑ absorbance with increasing serum dilutions</td>
<td>Decreased absorbance (wash before block)</td>
<td>II pg 48, III pg 13,19</td>
</tr>
<tr>
<td></td>
<td>Increased absorbance (block before wash)</td>
<td>III pg 34, 36</td>
</tr>
<tr>
<td>4. ↓ absorbance with increased oxLDLs spiked in serum</td>
<td>Decreased absorbance</td>
<td>III pg 12, 18</td>
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</table>

### ELISA for Autoantibodies

<table>
<thead>
<tr>
<th>Theoretical</th>
<th>Experimental</th>
<th>Pages in Notebook</th>
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</thead>
<tbody>
<tr>
<td>1. No interaction with BSA coat</td>
<td>Interaction, suggesting autoantibodies against BSA in serum</td>
<td>Notebook III pg 33</td>
</tr>
<tr>
<td>2. No interaction with LDLs coat</td>
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<td>III pg 33</td>
</tr>
<tr>
<td>3. ↓ absorbance with increased serum dilutions</td>
<td>↓ absorbance</td>
<td>III pg 32, 36</td>
</tr>
<tr>
<td></td>
<td>undiluted serum produces lower absorbance readings</td>
<td>III pg 19, 21</td>
</tr>
<tr>
<td>4. ↓ absorbance with ↑ oxLDLs added to serum</td>
<td>↑ absorbance</td>
<td>III pg 39</td>
</tr>
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Discussion

One of the most common methods to oxidize LDLs is incubation with CuSO₄ (8, 13, 25-27). This was the approach initially selected. The oxidation process can be divided into three phases, evident in Graph 1. During the lag phase, antioxidants contained in the LDLs, such as vitamin E, protect the LDLs from oxidation by reacting with the lipid peroxyl radicals (13). Once the vitamin E is depleted, PUFA are oxidized at an exponential rate forming lipid radicals. These lipid radicals can initiate further oxidation of PUFA (this is referred to as the propagation phase). As fatty acids are oxidized, the double bonds rearrange into a more stable conjugated diene form, which is measured as the increase in absorbance at 234 nm. During the decomposition phase the double bonds are broken, causing a decrease in absorbance, and oxidation products such as malondialdehyde are formed (1).

While the absorbances clearly suggested oxidation, Western Blotting failed to confirm the presence of oxLDLs, suggesting the primary antibody did not bind these oxidized LDLs. After many oxidation attempts with different concentrations of CuSO₄, it was finally determined that the antibody used was specific for LDLs oxidized using malondialdehyde rather than copper ions (Sigma technical support).

Once malondialdehyde was used to oxidize the LDLs, Western Blotting confirmed the presence of successfully oxidized LDLs. The mobility of oxLDLs was compared to that of native LDLs, with the oxLDLs traveling further across agarose gel toward the positively charged anode. LDLs that are oxidatively modified are more negatively charged (1). During the oxidation of LDLs, malondialdehyde (MDA) may covalently bind to the positively charged lysine group of apolipoprotein B, forming a Schiff base and giving the apolipoprotein a more negative charge and increased mobility through the gel (37). Perhaps this oxidative modification
is what scavenger receptors recognize (4). Increased mobility through the gel therefore indicates increased negativity (or increased charge/mass ratio) and suggests the oxidation of LDLs (1).

Western Blotting also verified that the primary antibody bound specifically to oxidized but not native LDLs. The specificity of this ELISA method permits the direct measurement of oxLDLs, while other common methods are only estimating oxLDLs indirectly. The specificity is demonstrated in Graph 4, where primary antibody bound selectively to oxLDLs and not to BSA or native LDLs.

Since oxLDLs are taken up rapidly by scavenger receptors (1, 18, 19, 24), it was unclear initially whether oxLDLs could be detected in serum (1, 24). Sensitive ELISA techniques, however, have confirmed the presence of oxLDLs (7, 21, 38) and autoantibodies against oxLDLs (8-11) in serum. This suggests that oxidation occurs in microenvironments of the arterial wall (12, 20) and/or that oxLDLs may leak back out into the circulation (1, 24). Furthermore, higher levels of oxLDLs and autoantibodies against oxLDLs are measured in undiluted serum compared to 1:10 and 1:100 diluted serum (see Graphs 6 and 9).

The ELISA method that detects autoantibodies against oxLDLs in serum initially gave some unexpected results. During this procedure, oxLDLs are coated on the plate, followed by washing with 1% BSA-PBS, blocking with 5% BSA-PBS, and then addition of serum samples. While the samples are incubated, the autoantibodies in the serum bind to the oxLDLs on the bottom of the plate. More dilute serum should generate lower absorbance readings, due to the presence of less autoantibodies, than more concentrated serum. However, early results repeatedly indicated that undiluted serum contained less autoantibodies than less concentrated, diluted serum (Graph 8). It was unclear whether the undiluted serum was producing artificially low absorbance values or the diluted serum was producing artificially high values. Either
something in the diluting agent (1% BSA-PBS) was binding to the plate and recognized by autoantibodies in the serum, thus increasing absorbances; or something in the undiluted serum, such as protein or other antibodies, was attaching to the plate and hindering binding of the autoantibodies. Undiluted serum contains more protein and antibodies that can adhere to the plate than diluted serum. If non-specific binding is occurring, then the buffer did not effectively block all open sites on the plate. Graph 13 illustrates the reduction of nonspecific binding when a blocking agent is added, observed as decreased absorbance readings. The higher absorbance values obtained when using TBS-skim milk for blocking suggest that it does not block open sites on the plate as effectively as BSA-PBS (Graph 12). If the blocking agent is not thoroughly binding all open sites on the plate, other antibodies present in the serum could attach and may potentially be recognized by the secondary antibody.

In an attempt to reduce non-specific binding, the order of the washing and blocking steps was reversed, so that blocking of open sites could occur prior to washing. This change resulted in increased absorbances for undiluted serum, which indicates the presence of more autoantibodies than diluted serum (Graph 9). The reversal allowed the effective blocking of open sites on the plate with 5% BSA-PBS immediately after excess antigen was removed. The original procedure called for wells to be rinsed with 1% BSA-PBS three times and then blocked with the 5% solution. It is possible that a layer of 1% BSA-PBS remained in the wells following the washing step and that this solution either diluted the blocking agent or prevented it from effectively binding to open sites on the plate, ultimately resulting in ineffective blocking.

The secondary antibody (alkaline phosphatase-conjugated mouse anti-human IgG) used when detecting autoantibodies in serum recognizes all human IgG antibodies. To verify the specificity of the antibody for IgG, an IgG-free serum was collected (Sigma Protocol, See Appendix). Serum was
run over a protein-A sepharose column and theoretically IgG bound to the column. The serum collected was free of IgG but presumably contained antibodies of other classes, such as IgE, IgA, IgM, and IgD. It is obvious from Graph 9 that the secondary antibody is specific for IgG.

Undiluted human serum is approximately 6% protein (39). Included in this fraction are antibodies to a variety of antigens including cow’s milk proteins and BSA (36). Therefore the blocking agent must be chosen carefully. If human serum contains antibodies to a blocking agent (such as BSA or skim milk), these antibodies may bind to the blocking agent attached to the plate. The secondary antibody would also recognize and bind to these autoantibodies. This would result in artificially high estimates of autoantibodies against oxLDLs. To avoid this problem serum samples were diluted in 1% BSA-PBS. Diluting with BSA permits any anti-BSA antibodies present in the serum to complex with the BSA, which would theoretically prevent binding of these antibodies to the BSA on the plate.

In the next experiment, serum was spiked with various amounts of oxLDLs to determine the effect on autoantibody binding. In theory, as more oxLDLs are added to the serum, more autoantibodies would complex with the added oxLDLs, and less autoantibodies would be available to bind to the plate. However, as more oxLDLs were added to the serum, the absorbance readings went up (Graph 11). This suggests that the added oxLDLs were binding to open sites on the plate and again indicates a failure of the blocking agent, or that the exogenous oxLDLs were binding to autoantibodies already to the plate, permitting another layer of autoantibodies to bind.

While this ELISA method detects LDLs that have been oxidatively modified with malondialdehyde, it does not detect Cu²⁺ oxidized LDLs. The fact that the antibody against
MDA-oxidized LDLs did not recognize copper-oxidized LDLs suggests the generation of different modifications to the LDLs depending on the method of oxidation. It is not clear which of these oxidatively modified forms is most representative of what happens in vivo. Advanced human atherosclerotic lesions do contain iron and copper ions (40) which may facilitate the oxidation of LDLs in the arterial wall. However, it is unclear whether they are present at concentrations large enough to actually induce oxidation (22). Malondialdehyde-modified LDLs have been isolated from atherosclerotic lesions of rabbits (38, 41) and humans (38). A variety of modifications may occur during in vivo oxidation, and oxLDLs have not yet been fully characterized.

It is probable that there are a number of substances in the body which can modify LDLs. Macrophages, smooth muscle cells, and endothelial cells, all of which are present in the arterial wall, can induce oxidation in vitro (42). In addition, there may be varying degrees of oxidation which take place as well. LDLs that are subjected to milder oxidation conditions (minimally-modified LDLs) are still recognized by the classic LDL receptor and not by scavenger receptors (42). However, minimally-modified LDLs (MM-LDLs) facilitate some initial steps which lead to the production of foam cells (22). Therefore, elevated levels of MM-LDLs may also increase an individual's risk for developing atherosclerosis.

Freshly oxidized LDLs and LDLs which had been oxidized earlier and stored at 4°C for two months were compared. Small differences in absorbances were apparent between wells that were coated with older versus freshly oxidized LDLs indicating that oxLDLs may change slightly over time. Therefore, within each assay, only one source of oxLDLs should be used.
Although the same procedure is followed each time the LDLs are oxidized, the procedure may generate products with varying degrees of oxidation. LDLs oxidized on different occasions could be tested by gel electrophoresis to determine their electrophoretic mobility compared to that of LDLs oxidized at other times. It is assumed that particular distances traveled through the gel could signify varying degrees of charge/size ratios and could indicate major or minor differences in the degree of oxidation of a given sample of LDLs.

The ELISA methods successfully detected different levels of both oxLDLs and autoantibodies against oxLDLs in various dilutions of human serum. Blocking and washing agents and serum and antibody dilutions which yield reproducible results were determined. However, there are some limitations that exist with the procedures. One of the problems with the measurement of oxLDLs in serum is the possibility that oxLDLs are taken up very rapidly from the circulation and that the concentration in the serum may not be representative of the extent of oxidation that has occurred in the arterial wall (1, 43). In addition, oxLDLs-autoantibody complexes may escape detection when assaying for the presence of oxLDLs. However, if the autoantibodies and the secondary antibody specific for oxLDLs target different epitopes on the oxLDLs, it may be possible to detect the complexes. It is clear that further characterization of oxLDLs and the interactions between oxLDLs and autoantibodies must be investigated.

For the comparison of data between research facilities, one standard ELISA procedure or procedures which produce comparable results must be adopted. Currently a variety of antibody and serum concentrations and blocking agents and substrates are used. It appears that this ELISA procedure can detect small quantities of oxLDLs and autoantibodies against oxLDLs in serum. Once this method is determined to generate reproducible and quantifiable data, serum samples from subjects can be analyzed.
Levels of oxLDLs and autoantibodies need to be compared with classic risk factors to interpret the significance of oxLDL levels and the magnitude of risk associated with oxLDLs. With this ELISA procedure, levels of oxLDLs in individuals could be monitored over time. This may allow for the screening of individuals who would not be recognized by a simple lipid profile; that is, their LDLs may not be elevated but they are at risk because of an increased percentage of LDLs which are oxidized. Once this risk is identified in an individual, lifestyle changes could be made to decrease their level of oxLDLs and prevent further oxidation from occurring; ultimately decreasing their risk of developing atherosclerosis.

Remaining Questions and Future Directions

Although the assays have been successful with the antibody concentrations chose, it may be beneficial to further test antibody dilutions. If a more dilute antibody concentration produces similar results, this would be more cost effective. Additional experimentation should also be done with blocking agents. While the effectiveness of 5% BSA-PBS was compared to that of TBS-skim milk, it would be beneficial to test various concentrations of BSA to determine if an optimal amount of blocking can be achieved that would minimize non-specific binding.

An ELISA should be done to determine why adding increasing amounts of oxLDLs to the serum did not result in decreased autoantibody binding to the plate (Graph 11). In addition, the collection of IgG-free serum should also be repeated. The absence of IgG and the presence of other classes of immunoglobulins was assumed after running the serum through the column. A dot blot test could be done to determine the presence or absence of IgG, and the presence of other classes of immunoglobulins could be determined with secondary antibodies specific for these classes.
Once optimal blocking is achieved, repeatable results must be obtained. Then a standard curve may be devised by which known concentrations of oxLDLs correspond to absorbance values. This would allow for absorbance values to be equated with concentrations of oxLDLs in the serum.

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Appendix

Detailed Procedures

**Gel Electrophoresis**

0.5% agarose gel was made by dissolving 0.500 g of agarose in 100 mL Tris-Tricene (4.847 g Tris, 2.149 g Tricene, and H₂O up to a volume of 1 L). The agarose solution was heated in a microwave until the solution was clear (approximately 1.5 minutes on high). Hot agarose solution was allowed to cool approximately 5 minutes before pouring onto a Submarine Gel Electrophoresis Plate. 25 mL of agarose solution was pipetted onto the plate via a warm graduated glass pipet. The well-separator was inserted and the gel was allowed to solidify for 45 minutes to an hour.

15 μL samples (including 5 μL of bromosucrose solution, 0.1 g Bromophenol Blue, 0.822 g sucrose, and 20 mL H₂O), were loaded in the wells and Tris-Tricene was used as the gel buffer. Gels were run at 56 mA until the dye front nearly reached the end of the gel plate (approximately 45 minutes to an hour).

**Copper Oxidation**

5 μL of LDLs (10 mg/mL) (CalBiochem), 33 μL of 100 μM CuSO₄, and 962 mL PBS (final CuSO₄ concentration of 3.32 μM and LDL concentration of 0.05 mg/mL) incubated at 37°C. Absorbance was read at 234 nm every 15 minutes against a blank of water and a control of 3.32 μM CuSO₄ in PBS in a Genesys Spectrophotometer. 40 μL aliquots were collected and quenched with 5 μL of 0.1 M EDTA.
Dialysis of MDA-modified LDL

Dialysis Buffer: 4.383 g NaCl, 0.05 g disodium EDTA, 50 mL sodium phosphate buffer, H$_2$O to a final volume of 500 mL.

A Slide-A-lyzer dialysis cassette (Pierce) was submerged in dialysis buffer for 30 seconds. After submersion, sample was injected into cassette via syringe. The needle was carefully inserted into the cassette to avoid puncturing the cassette membrane. Once the sample was injected, excess air was removed from the cassette via syringe. The cassette was placed in a buoy in 200 mL dialysis buffer in a medium-sized beaker covered with saran wrap at 4 °C for sixteen hours. Dialysis buffer was changed once during this time.

Column for collection of IgG-free serum (Sigma Protocol)

Protein A Sepharose (Sigma)
Buffer A: (2.402 g NaH$_2$PO$_4$, 8.799 g NaCl, 1 L H$_2$O) @ pH 8.0
Buffer B: (24.3 mL 0.1 M citric acid, 25.7 mL 0.2 M Na$_2$HPO$_4$, 50 mL H$_2$O) @ pH 4.0

1 mL of Buffer A was added to Protein A and allowed to remain at room temperature for 30 minutes. Protein A Resin was transferred into a syringe equipped with a three-way stop-cock. The column was washed with 20 column volumes (CV) of Buffer A (20 mL), and then 2 mL of serum was added. The column was washed with 10 CV of Buffer A, and IgG-free serum fractions were collected in bullet tubes via a fraction collector. IgG was collected off the column by adding 3 CV of Buffer B. This was followed by 25 CV of Buffer A. To collect the Protein A sepharose for reuse, 1 mL of Buffer A was injected via syringe into the stop-cock to force the resin out of the column and into a 10 mL beaker. Protein A was transferred into a storage bottle and ethanol was added to a concentration of 20% to preserve the Protein A.
Works Cited


PROJECT TITLE: The Development of ELISA Methods for the Measurement of Oxidized LDL and Autoantibodies Against Oxidized LDL in Human Serum.

Approved by:

Amy Olson, Professor of Nutrition, Chair of Nutrition Department

Henry Jakubowski, Associate Professor of Chemistry

Ingrid Anderson, OSB, Professor of Nutrition

Margaret Cook, Director, Honors Thesis Program

Charles Bobertz, Director, Honors Program