Induction of T-Cell Activation by Oxidized Low Density Lipoprotein

Johan Frostegård, Ruihua Wu, Ricardo Giscombe, Göran Holm, Ann Kari Lefvert, and Jan Nilsson

Oxidation and scavenger receptor-mediated uptake of low density lipoprotein (LDL) in intimal macrophages are believed to be key events in the development of atherosclerosis. We report here that oxidized LDL increases DNA synthesis, expression of HLA-DR, and interleukin-1β receptors in T cells. The stimulatory effect of oxidized LDL was not due to a direct effect on T cells but required the presence of monocytes. Oxidized LDL also stimulated the release of interleukin-1β from monocytes. The maximal effect of oxidized LDL on T-cell activation and interleukin-1β release occurred at a concentration of 1 μg/ml. Native LDL also had the capacity to activate T cells, although only at higher concentrations. The stimulatory effect of both native and oxidized LDL was inhibited by superoxide dismutase. Monocytes as well as T cells were found to have the ability to oxidize LDL, suggesting that the stimulatory effect of native LDL may arise as a result of LDL oxidation during incubation with monocytes and T cells. The results suggest that oxidized LDL may activate T cells in atherosclerotic lesions. (Arteriosclerosis and Thrombosis 1992;12:461–467)

KEYWORDS • atherosclerosis • lymphocytes • hypercholesterolemia • interleukins • monocytes

Macrophages, smooth muscle cells, endothelial cells, and T lymphocytes are the most frequent cell types present in atherosclerotic lesions. Interactions between these cells and lipoproteins are believed to be of major importance for the development of atherosclerosis. Considerable interest has been focused on the possible role of oxidized low density lipoprotein (LDL) in the initiation of early lesions.1 The concept of oxidized LDL as a key molecule in atherogenesis originates from the finding that macrophages, a cell type with few receptors for native LDL, bind and take up oxidized LDL via a specific family of receptors that are referred to as scavenger receptors.2 Two subclasses of scavenger receptors have recently been cloned and found to express considerable homology. Both receptors have a trimeric structure and contain short collagen-like repeats.3,4 Several lines of evidence have indicated that oxidation of LDL promotes development of atherosclerosis. Oxidized LDL is a chemotactant for monocytes,2 enhances monocyte adhesion to endothelial cells, and initiates monocyte differentiation into tissue-bound macrophages.5 Oxidized LDL is also highly cytotoxic, especially to endothelial cells,6 and removal of cytotoxic oxidized LDL may be one of the most important functions of the macrophage scavenger receptors. Scavenger receptors are also present on the surface of endothelial cells,8 but the pathophysiological role of these receptors is less clear. Oxidized LDL has been found to enhance the adhesive properties of endothelial cells,9 and LDL modified by acetylation has been found to decrease the secretion of platelet-derived growth factor (PDGF) from endothelial cells.10

Although cell-culture experiments have clearly suggested a role for oxidized LDL in atherogenesis, much less is known about the in vivo effects of LDL oxidation. Using immunocytochemistry, several groups have been able to demonstrate the presence of oxidized LDL in atherosclerotic lesions and to elute oxidized LDL from lesions.11 Treatment of Watanabe heritable hyperlipidemic rabbits with probucol, a lipid-lowering drug with antioxidant properties, has been found to prevent development of atherosclerosis much more effectively than other drugs with similar lipid-lowering effects.12,13 Furthermore, increased levels of plasma lipid peroxides have been demonstrated in patients with ischemic heart disease and peripheral artery disease.14

The mechanism by which oxidation of LDL occurs in vivo remains to be determined. In vitro, LDL oxidation can be accomplished by exposure to copper ions. However, cultured endothelial cells,15 monocytes/macrophages,10 and smooth muscle cells17 also oxidatively modify LDL, suggesting the existence of more physiological forms of oxidative modification.

Little is still known concerning the effect of LDL oxidation on T-cell function. In the present article we report that exposure of peripheral blood mononuclear cells (PBMCs) to low concentrations of oxidized LDL results in T-cell activation. The possible implications for oxidized LDL-mediated activation of T cells in atherogenesis is discussed.
Methods

Preparation of Low Density Lipoprotein

PBMNs were isolated from human buffy coats. Theuffy coat was diluted in phosphate-buffered saline (PBS) at a ratio of 1:4, layered onto Ficoll-Hypaque (Pharma-cia, Uppsala, Sweden), and centrifuged for 20 minutes at 1,500g. Interface cells were washed twice in PBS. They were then counted and resuspended in RPMI-1640 medium supplemented with 50 µg/ml gentamicin and 10% fetal calf serum at a cell concentration of 1x10^6 cells/ml. The PBMN suspensions contained approximately 10% monocytes, 30–40% T cells, and 50–60% B cells.

Then PBMNs were isolated from human buffy coats. First, PBMNs were isolated as described above. After incubating the cells for 1 hour at 37°C in 60-mm petri dishes, nonadherent cells were removed by washing the plates twice in PBS. The adherent cells were scraped off the dishes using a rubber policeman. They were then washed and seeded at a concentration of 1x10^6 cells/ml in 12-multiwell plates. Native or oxidized LDL was added after approximately 1 hour when the cells had become adherent. Cell viability was determined by Trypan blue dye exclusion and exceeded 90% in all experiments.

T cells were isolated from human buffy coats. PBMNs were isolated as described above; then 10 ml sheep red blood cells was centrifuged for 5 minutes at 800g. A 0.1-ml volume of the pellet consisting of packed sheep red blood cells was added to PBS containing 1% neuraminidase and incubated for 30 minutes at 37°C. The sheep red blood cells were washed three times in PBS and mixed with 4 ml PBMNs to a final concentration of 4x10^6 cells/ml. This suspension was gently shaken and put on ice overnight. It was then layered onto Ficoll-Hypaque and centrifuged for 20 minutes at 1,500g. The sediment was washed three times in PBS. During the experiments the cells were kept in RPMI-1640 medium containing 10% heat-inactivated human serum pooled from 10 normal donors (human AB serum) and 50 µg/ml gentamicin. Over 90% of the cells in the preparation were T cells, as assessed by FACS-Scan (Becton Dickinson, Mountain View, Calif.) analysis of CD3 expression.

Oxidation of Low Density Lipoprotein

LDL was dialyzed three times against PBS for 24 hours. Copper oxidation of LDL was performed by incubating 0.2 mg/ml LDL in F-10 medium containing 10 µM CuSO_4 overnight at 37°C. CuSO_4 was not removed after the oxidation, but in concentrations up to 1 mM, CuSO_4 was without effect on concanavalin A (con A)–induced PBMN DNA synthesis. To analyze cellular oxidation, PBMNs, monocytes, and T cells were prepared as described above and kept in RPMI-1640 medium containing 1% human AB serum. LDL was added to the cells at the indicated concentrations for 24 hours at 37°C. The LDL-containing supernatants were then harvested and immediately analyzed for the presence of lipid peroxides. Iodination of LDL and analysis of LDL degradation in mouse peritoneal macrophages were performed as described earlier. The presence of endotoxins in the lipoprotein preparations was analyzed using the Limulus assay (Kabi, Stockholm, Sweden). All endotoxin levels were below 2.5 pg/mg LDL protein in the stock solutions and below 5 pg/mg LDL protein in the test samples. There was no difference in endotoxin levels between native and oxidized LDL.

Determination of Lipid Peroxide Content

The lipid peroxide contents of oxidized and native LDL were determined by analyzing thiobarbituric acid-reactive (TBAR) substances and expressing them as malondialdehyde (MDA) equivalents. Five hundred microliters of the LDL preparation (200 µg/ml) was mixed with 1 ml MDA reagent (10 ml 30% [vol/vol] trichloroacetic acid, 1.25 ml 4 M HCl, 112 µl 2% [vol/vol] butylated hydroxytoluene, and 0.755% thiobarbituric acid). The samples were heated in boiling water for 20 minutes, cooled, and then centrifuged at 12,000g for 2 minutes. The optical density was read at 532 nm. Fresh tetramethoxypropane, which produces MDA, was used as a standard.

Determination of DNA Synthesis

PBMNs were prepared as described above and suspended in RPMI-1640 medium with addition of 10% heat-inactivated human AB serum at a cell concentration of 2x10^6 cells/ml. The cells were then seeded in 96-well plates, with 100 µl cell suspension/well, and incubated with 2 µCi/ml [3H]thymidine for 72 hours at 37°C. DNA was precipitated in glass fiber filters by means of an automatic cell harvester, and the amount of incorporated [3H] was determined in a liquid scintillation counter.

Expression of Surface Antigens

The monoclonal fluorescein isothiocyanate–conjugated CD3, phycoerythrin-conjugated HLA-DR, and phycoerythrin-conjugated CD25 antibodies were purchased from Becton Dickinson. PBMNs were washed three times in PBS/0.02% NaN_3. The pellet was then gently suspended, the monoclonal antibodies were added to the cell suspension, and the suspension was placed on ice under cover for 30 minutes. Thereafter, the cells were washed twice in PBS, and the expression of surface antigens was determined in an FACS-Scan (Becton Dickinson).

Determination of Interleukin-1β

PBMNs were prepared as described above and suspended in RPMI-1640 medium with addition of 10% human AB serum at a concentration of 10^6 cells/ml. The cells were incubated with native or oxidized LDL for 24 hours. The concentration of interleukin-1β (IL-1β) in the
TABLE 1. Effect of Oxidation on Thiobarbituric Acid-Reactive Content and Macrophage Degradation of Low Density Lipoprotein

<table>
<thead>
<tr>
<th></th>
<th>TBAR material</th>
<th>Macrophage degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(malondialdehyde equivalents/mg protein)</td>
<td>(ng/mg cell protein)</td>
</tr>
<tr>
<td>Native LDL</td>
<td>1.8±0.15</td>
<td>0.14±0.02</td>
</tr>
<tr>
<td>Cu²⁺-oxidized LDL</td>
<td>41.0±2.5</td>
<td>1.53±0.24</td>
</tr>
</tbody>
</table>

Low density lipoprotein (LDL) was oxidized by exposure to 10 μM CuSO₄ in F-10 medium for 16 hours at 37°C. Analysis of thiobarbituric acid-reactive content and macrophage degradation was performed as described in "Methods." Each value represents mean±SEM of six determinations.

Results

Low Density Lipoprotein Oxidation

Exposure of native LDL to 10 μM Cu²⁺ for 16 hours at 37°C resulted in a 20-fold increase in the amount of TBAR material present in the LDL preparation (Table 1) and an increased mobility during agarose gel electrophoresis (data not shown). Exposure of native LDL to 10 μM Cu²⁺ also led to an increased rate of degradation in cultured mouse peritoneal macrophages (Table 1). Incubation of native LDL in RPMI-1640 medium alone for 24 hours at 37°C resulted in a minor increase in TBAR content of the lipoprotein preparation. However, incubation of LDL together with PBMs in RPMI-1640 medium for 24 hours resulted in a further fourfold increase (<0.001) in the amount of TBAR material (Figure 1). The ability of PBMs to oxidize LDL was dependent on cell concentration, reaching a maximal effect at a density of approximately 5×10⁵ cells/ml. To analyze which cell type in the PBMN preparation was responsible for the oxidation of LDL, native LDL was incubated in the presence of isolated monocytes and T cells. The ability of T cells to oxidize LDL was found to be similar to that of PBMs and monocytes (Figure 1).

DNA Synthesis in Peripheral Blood Mononuclear Cells Exposed to Native or Oxidized Low Density Lipoprotein

In cells exposed to 1 μg/ml oxidized LDL for 72 hours, a fivefold increase (<0.001) in DNA synthesis was observed (Figure 2). At higher concentrations the effect of oxidized LDL declined, reaching the baseline value at a concentration of 10 μg/ml. Cu²⁺, in the concentrations present in the preparations of oxidized LDL tested, did not affect DNA synthesis in PBMs (data not shown). Native LDL was not as effective as oxidized LDL in stimulating PBMN DNA synthesis. At a concentration of 1 μg/ml, native LDL was almost without effect on the rate of DNA synthesis, whereas a 164±15% increase (<0.05) was observed in cells incubated with 10 μg/ml native LDL (Figure 2). Exposure of isolated T cells and monocytes to native or oxidized LDL did not result in any significant change in the rate of DNA synthesis (Figure 3). The stimulatory effect of 20 μg/ml Con A, a known polyclonal T-cell activator, was 10–15 times higher than the stimulatory effect of LDL or oxidized LDL. To study the relative importance of monocyte-derived cytokines in lipoprotein-induced PBMN DNA synthesis, isolated monocytes were exposed to medium containing native or oxidized LDL for 2 hours. The lipoprotein-containing medium was then determined using an enzyme-linked immunosorbent assay (Eurogenetics, Brussels, Belgium), using a monoclonal antibody specific for IL-1β.

![Figure 1](http://www.ahajournals.org/doi/figure/10.1161/01.ATV.81.3.463)

![Figure 2](http://www.ahajournals.org/doi/figure/10.1161/01.ATV.81.3.463)
Figure 3. Line plot showing effect of oxidized low density lipoprotein (LDL) on DNA synthesis in isolated T cells and monocytes. Isolated T cells (o) and monocytes (•) were exposed to the indicated concentrations of oxidized LDL in RPMI-1640 medium with 10% human AB serum in 96-well plates for 72 hours. [3H]thymidine (2 μCi/ml) was added during the last 6 hours of incubation. DNA was precipitated in glass fiber filters and the amount of incorporated [3H]thymidine determined in a liquid scintillation counter. Each value represents the mean of four determinations (SD<15%).

Figure 4. Line plot showing effect of oxidized low density lipoprotein (LDL) on release of T-cell mitogens from monocytes. Monocytes were exposed to oxidized (•) or native (o) LDL for 24 hours. Lipoproteins were then removed, and the cells were kept in a lipoprotein-free medium for another 24 hours. The conditioned medium was recovered and its mitogenic effect on isolated T cells determined. Conditioned medium from cells exposed to oxidized LDL stimulated T-cell DNA synthesis in a dose-dependent manner, whereas medium from cells exposed to native LDL was without effect (Figure 4).

Effect of Superoxide Dismutase on Peripheral Blood Mononuclear Cell DNA Synthesis

PBMNs were preincubated with superoxide dismutase (SOD) at the indicated concentrations for 1 hour and then exposed to 1 μg/ml oxidized LDL or 10 μg/ml native LDL in the presence of SOD for 72 hours. Values are expressed as percentages of lipoprotein-induced DNA synthesis in the absence of SOD. In this experiment the rate of DNA synthesis in cultures incubated in lipoprotein-free medium was about 15% of that in cultures exposed to 1 μg/ml oxidized LDL or 10 μg/ml native LDL. SOD was found to block almost 90% of the stimulatory effect of native LDL and almost 60% of the stimulatory effect of oxidized LDL on T-cell activation (Figure 5).

Effect of Chloroquine on Peripheral Blood Mononuclear Cell DNA Synthesis

PBMNs were stimulated with 1 μg/ml oxidized LDL or 20 μg/ml con A in the presence of different concentrations of the lysosomotropic drug chloroquine for 72 hours. At a concentration of 1 μg/ml, chloroquine almost completely inhibited the mitogenic effect of oxidized LDL, whereas essentially no effect on con A–induced DNA synthesis was observed at this concentration of chloroquine. At higher concentrations chloroquine inhibited both con A– and oxidized LDL–induced DNA synthesis (Figure 6).

Expression of Surface Antigens on Peripheral Blood Mononuclear Cells Exposed to Native and Oxidized Low Density Lipoprotein

Expression of IL-2 receptors on T cells in the PBMN preparations was analyzed by determining the fraction of CD25-positive cells. Exposure of PBMNs to low concentrations of oxidized LDL (up to 5 μg/ml) for 24 hours resulted in a significant increase in the fraction of CD25-positive cells (p<0.001, Figure 7). At higher concentrations the fraction of CD25-positive cells decreased, and in
FIGURE 6. Semilog plot showing effect of chloroquine on oxidized low density lipoprotein (LDL) and concanavalin (con) A–induced peripheral blood mononuclear cell (PBMC) DNA synthesis. PBMCs were incubated with 1 μg/ml oxidized LDL (●) or 20 μg/ml con A (○) and the indicated concentrations of chloroquine for 72 hours in RPMI-1640 medium with 10% human AB serum in 96-well plates. [3H]thymidine (2 μCi/ml) was added during the last 6 hours of incubation. DNA was precipitated in glass fiber filters and the amount of incorporated [3H]thymidine determined in a liquid scintillation counter. Each value represents the mean of four determinations (SD<15%).

FIGURE 7. Line plot showing effect of native and oxidized low density lipoprotein (LDL) on the expression of CD25 surface antigen on T cells. Peripheral blood mononuclear cells were grown for 24 hours with addition of native (●) or oxidized (○) LDL at the indicated concentrations. They were then exposed to fluorescein-conjugated CD3 and phycoerythrin-conjugated HLA-DR monoclonal antibodies for 30 minutes. The fluorescence of CD3-positive cells was then determined in a FACS-Scan.

FIGURE 8. Line plot showing effect of native and oxidized LDL on the expression of HLA-DR surface antigen on T cells. Peripheral blood mononuclear cells were grown for 48 hours in presence of native (●) or oxidized (○) LDL at the indicated concentrations. They were then exposed to fluorescein-conjugated CD3 and phycoerythrin-conjugated HLA-DR monoclonal antibodies for 30 minutes. The fluorescence of CD3-positive cells was then determined in an FACS-Scan.

expression, higher concentrations of oxidized LDL resulted in a decrease in the fraction of HLA-DR-positive T cells (Figure 8). Native LDL also increased the fraction of HLA-DR–expressing CD3-positive cells, but a concentration of 10 μg/ml was required for a maximal effect. No effect of oxidized LDL or native LDL on HLA-DR or CD25 expression was found in isolated T cells (data not shown).

Interleukin-1β Production by Peripheral Blood Mononuclear Cells Exposed to Native and Oxidized Low Density Lipoprotein

The basal level of IL-1β production in the PBMN preparation was 320 pg/10^6 cells. Exposure of the cells to 1 μg/ml oxidized LDL gave rise to a 139±9.5% increase (p<0.001) in IL-1β synthesis, whereas no effect was observed at higher concentrations of oxidized LDL. Native LDL did not influence IL-1β production in concentrations up to 10 μg/ml (Figure 9).

Discussion

The present findings demonstrate that exposure of PBMs to low concentrations of oxidized LDL activates T cells, as manifested by an increased DNA synthesis and an increased expression of HLA-DR antigen and IL-2 receptors. The stimulatory effect of oxidized LDL is not due to a direct effect of the modified lipoprotein on T cells but is the result of a monocyte-dependent process. Exposure of isolated monocytes to oxidized LDL for 2 hours resulted in the release of a T-cell mitogen. Oxidized LDL, in concentrations resulting in T-cell activation, also stimulated the release of IL-1 from monocytes. Hence, it is possible that in the presence of oxidized LDL, monocytes/macrophages may initiate T-cell activation by releasing IL-1β. The important question as to whether the stimulatory effect of oxidized LDL on PBMN DNA synthesis involves HLA-DR–dependent antigen presentation remains to be answered. It has been reported that chloroquine exerts a highly specific inhibitory effect on the presentation of large antigens by macrophages.22 The present finding
The presence of endotoxins. T-cell activation by oxidized LDL (and LDL at higher concentrations) was partly inhibited because no differences were found in endotoxin levels between native and oxidized LDL preparations, it is unlikely that the additional T-cell-activating capacity obtained after oxidation can be explained by the presence of endotoxins. T-cell activation by oxidized LDL (and LDL at higher concentrations) was partly inhibited by SOD. In contrast, SOD had no effect on con A–induced T-cell activation, further supporting the notion that the stimulatory effects of oxidized LDL are not due to endotoxin contamination of the lipoprotein preparation. A third possible explanation is that the native LDL preparation contained minor amounts of oxidized LDL. However, the finding that native LDL contained very low levels of TBAR material and demonstrated normal mobility on agarose gel electrophoresis argues against any substantial oxidation occurring during lipoprotein isolation. The most probable explanation is that oxidation of native LDL takes place during the exposure to PBMsN, as both monocytes and T cells were found to have the capacity to oxidize LDL in vitro.

Earlier studies have demonstrated that LDL may exert a number of immunoregulatory actions. At low concentrations native LDL has been found to enhance DNA synthesis in mitogen-stimulated lymphocytes when the experiments were carried out under serum-free conditions. This phenomenon may be due to a basal cholesterol (and possibly also other lipids) requirement of proliferating lymphocytes. In contrast, higher concentrations of LDL were found to inhibit the mitogen response. An inhibitory effect of LDL on T-cell function is also observed when cells are grown in the presence of serum. Nakayasu and coworkers have reported that the inhibitory effect of LDL is not due to a direct effect on T cells but rather to an effect on accessory cells.

Recent immunocytochemical analyses have demonstrated that T cells constitute an important component of human atherosclerotic plaques. In a study of human carotid lesions, Hansson and coworkers reported that 34% of the T cells expressed HLA-DR and 6% showed positive staining for the IL-2 receptor. This expression pattern is similar to that reported in chronic inflammatory conditions and demonstrates that a substantial fraction of the T cells in human atherosclerotic plaques are in an activated state.

The experimental data presently available indicate that the role of T cells in atherogenesis is complex. In rats treatment with the T-cell inhibitor cyclosporin A significantly reduces arterial intimal proliferation after mechanical injury, suggesting that activated T cells either release growth factors for smooth muscle cells or induce other cells to do so. Indeed, several T-cell products are potent macrophage activators. Activated macrophages produce PDGF, which is a potent mitogen for smooth muscle cells. Recently, Ross and coworkers were able to demonstrate PDGF-containing macrophages in all phases of atherogenesis. Interferon gamma and transforming growth factor-β (TGF-β), which are secretory products of activated T cells, inhibit smooth muscle growth in vitro. TGF-β is also known to be a potent stimulator of connective matrix synthesis. It is possible that TGF-β released from T cells inhibits the proliferation of smooth muscle cells at the same time as it promotes local collagen synthesis. The latter process may decrease the risk for plaque rupture and the consequent acute ischemic complications. The presence of activated T cells in atherosclerotic plaques may also represent signs of an immune response. Palinski and coworkers were recently able to demonstrate the presence of antibodies against oxidized LDL in the sera from both healthy controls and patients suffering from...
cardiovascular disease. Whether these antibodies have developed as a result of an immune reaction against oxidized lipids in arterial tissue remains to be clarified.

Several lines of evidence have suggested that lipoprotein oxidation plays an important role in the development of atherosclerosis. Oxidized LDL may promote adhesion of monocytes to the endothelium and stimulate monocyte migration, differentiation of monocytes into tissue-bound macrophages, and development of lipid-rich foam cells.1 The present results demonstrate that oxidized LDL may activate T cells and stimulate release of IL-1 from monocytes. In theory this process may both promote and inhibit the further development of the atherosclerotic plaque. T-cell–produced cytokines have the capacity to activate macrophages to secrete inflammatory mediators and growth factors for smooth muscle cells, resulting in enhanced plaque growth. In contrast, T-cell products may also have a direct growth-inhibitory effect on smooth muscle cells, thus inhibiting further growth of the plaque. Clearly, much further work is required before the role of activated T cells in atherogenesis can be evaluated.

Acknowledgments
The authors thank Dr. Kerstin Carlson for preparation of LDL and Dr. Jan Regnström for determination of macrophage degradation of LDL.

References
13. Carew TE, Schwenke DC, Steinberg D: Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: Evidence that antioxidants in vivo can selectively inhibit low density degradation in macrophage-rich fatty streaks and slow progression of atherosclerosis. Proc Natl Acad Sci U S A 1987;84:7725–7729
berg D: Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc Natl Acad Sci U S A 1984;81:3885–3887
Induction of T-cell activation by oxidized low density lipoprotein.
J Frostegård, R Wu, R Giscombe, G Holm, A K Lefvert and J Nilsson

doi: 10.1161/01.ATV.12.4.461
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/12/4/461

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/