Oxidized LDL Can Induce Macrophage Survival, DNA Synthesis, and Enhanced Proliferative Response to CSF-1 and GM-CSF

John A. Hamilton, Damian Myers, Wendy Jessup, Fiona Cochrane, Robert Byrne, Genevieve Whitty, Suzanne Moss

Abstract—Modification of low density lipoprotein (LDL), eg, by oxidation, has been proposed as being important for the formation of foam cells and therefore for the development of atherosclerotic plaques. There are a number of reports showing that macrophage-derived foam cells can proliferate in both human and animal lesions, particularly in the early phase of the disease and possibly involving macrophage-colony stimulating factor (M-CSF, or CSF-1). We studied the in vitro effects of oxidized LDL (ox-LDL) on murine bone marrow–derived macrophages (BMMs), a cell population with a high proliferative capacity in vitro in response to CSF-1 and granulocyte macrophage-colony stimulating factor (GM-CSF); the effects were dependent on the degree of LDL oxidation. For CSF-1, a synergistic effect was noticeable at suboptimal doses. The effect of ox-LDL occurred even in the absence of endogenous CSF-1 or GM-CSF. Our findings suggest that ox-LDL, and possibly other modified forms of LDL, could maintain macrophage (and foam cell) survival and therefore lengthen their tenure in a plaque; the modified LDL could also cause local macrophage proliferation or “prime” them so that they could proliferate better in response to CSF-1 (and GM-CSF) concentrations that may be present in the atheroma. (Arterioscler Thromb Vasc Biol. 1999;19:98-105.)

Key Words: oxidized LDL ■ macrophages ■ proliferation ■ colony stimulating factor-1 ■ granulocyte macrophage-colony stimulating factor

LDL, the major cholesterol-carrying lipoprotein in blood, has long been implicated in the processes leading to coronary heart disease. Evidence supports the notion that LDL becomes fully atherogenic only after it has been modified in some way. There are data indicating that LDL oxidation can occur in vivo, and oxidized LDL (ox-LDL) has a number of potentially atherogenic properties, of which the best known is accelerated endocytosis by macrophages via the so-called scavenger receptors. Cholesterol ester–filled macrophages, or “foam” cells, are an early and prominent feature of atherosclerotic lesions, which in turn exhibit many features of a chronic inflammatory reaction.

It is still unclear which factors attract monocytes into the intima in the atheroma and which factors play important roles in controlling the subsequent differentiation into macrophages and foam cells. Colony stimulating factors (CSFs) regulate the survival, proliferation, and differentiation of hemopoietic progenitor cells into mature cells. Two such CSFs are macrophage-CSF (M-CSF, or CSF-1), which mediates the clonal proliferation and differentiation of progenitors into monocytes/macrophages, and granulocyte macrophage-CSF (GM-CSF), which generates both granulocytes and macrophages by similar processes. Both CSFs can also act on the mature cells in these lineages, making it likely that these CSFs have a role in inflammatory processes.5,3 CSF-1 circulates in the blood, and both it and its receptor (c-Fms) have been detected in atherosclerotic lesions. Vascular endothelial and smooth muscle cells produce CSF-1 and GM-CSF in vitro in response to a wide range of stimuli, including modified LDL; also CSF-1 enhances macrophage scavenger receptor expression and function in vitro as well as cell adhesion. These findings have led to the suggestion that CSF-1 and GM-CSF production in the atheromatous plaque microenvironment could promote the recruitment and retention of mononuclear phagocytes and subsequent foam cell formation.6–12 However, CSF-1 can also lower plasma cholesterol levels.13,14

Evidence has been mounting that macrophage-derived foam cells are able to proliferate, particularly in situ in the early stages of lesion development in humans and rabbits. For example, Villaschi and Spagnoli reported that the thymidine labeling in plaques was “almost exclusively in...
focal infiltrates of foam cells and monocyte-like cells.” In early human lesions it was shown recently that the vast majority of proliferating cell nuclear antigen–positive cells were monocytes/macrophages (and/or lymphocytes) but not smooth muscle cells. Both CSF-1 and GM-CSF expression is associated with macrophage proliferation in progressing and regressing rabbit atheromatous lesions. It is possible that plaque macrophages might themselves be proliferating under the influence of, eg, CSF-1, and that 2 pathways leading to an increase in intimal macrophage mass appear to exist: (1) migration of monocytes from the blood into the intima and (2) proliferation of macrophages within the plaque. However, it has also been suggested that ox-LDL is responsible for foam cell death.

Murine bone marrow–derived macrophages (BMMs) are an easily obtainable and homogeneous normal cell population that has an absolute requirement for a growth factor such as CSF-1 for its survival and proliferation. As such, these cells are useful for the study of the survival and proliferative responses to CSFs. We report here that at certain concentrations, ox-LDL can induce BMM survival, DNA synthesis, and an enhanced proliferative response to CSF-1 and GM-CSF.

Methods

Mice

Cells were obtained from male or female CBA mice as described before.

Bone Marrow–Derived Macrophages

BMMs were generated as adherent cells from their nonadherent progenitors in bone marrow as described before and grown to confluence in 24-well plates (Nunc) for 5 to 6 days in RPMI supplemented with 5×10⁻² mol/L 2-mercaptoethanol, 20 mmol/L HEPES, 15% FCS, and 20% L cell–conditioned medium (a crude source of CSF-1). The BMMs are a relatively pure and homogeneous population with ≥95% of the adherent cells binding CSF-1. Cells were usually prepared for experiments by washing twice with PBS and recultured in growth medium without L cell–conditioned medium. BMMs at this stage were usually “starved” of growth factor for 24 hours before use to render the cells quiescent. These quiescent cells were used for most of the experiments described herein. In some experiments, the growth medium was removed just before commencement of the experiment.

DNA Synthesis

DNA synthesis was measured as the incorporation of [methyl-³H]thymidine (Tdr) (2 μCi/mL). Uptake was stopped by removal of the culture medium and solubilization in 0.2 mol/L NaOH, and the incorporation of label into trichloroacetic acid–precipitable material was recovered using an Inotech cell harvester (Berthold-Australia). Incorporated radioactivity was counted on a Digital Autoradiograph

Flow Cytometry

BMMs were harvested by gentle scraping, centrifuged, and resuspended in PBS. To a 200-μL aliquot containing 2×10⁵ cells was added 50 μL of stock staining solution comprising 250 μg/mL propidium iodide (Pi; Sigma Chemical Co), 5 mg/mL RNase (EC 3.1.27.5; Sigma), and 1% Triton X-100 in distilled water. After being stained with PI the cells were incubated in the dark at 4°C for a minimum of 3 hours, after which PI fluorescence was measured using a fluorescence-activated cell sorter (FACS Calibur flow cytometer, Becton Dickinson). Cell cycle analysis was performed on gated, singlet populations by using ModFit LT cell cycle analysis software (Verity Software House, Inc). Acquisition was restricted to 20 000 events for each sample.

BMM Numbers

For quantification of the number of BMMs in culture, the medium was removed and the cells gently scraped; viable cells were counted in a hemacytometer with the use of trypan blue exclusion.

LDL Preparation

Human LDL was isolated from healthy, fasting volunteers in the presence of 3 mmol/L EDTA by discontinuous density-gradient ultracentrifugation in the density range ρ=1.02 to 1.05, as previously described. The isolated LDL was sterilized by membrane filtration (0.45 μm) and stored in the dark at 4°C under N₂. LDL preparations were used within 1 week of isolation. All materials and solutions were pretreated to remove endotoxin.

LDL Modifications

Oxidation

LDL was desalted into PBS by passage over 2× Sephadex G-25 (PD-10; Pharmacia) in series to remove KBr and EDTA. Copper oxidation was achieved by incubating LDL (1 mg protein per mL) in PBS with a sterile solution of CuCl₂ (final concentration, 20 μmol/L) at 37°C for 24 hours. Oxidation was arrested by addition of serum or by dialysis against PBS to remove excess CuCl₂, and the material was used within 7 to 14 days of preparation.

Acetylation

LDL (3 to 4 mg protein per mL) was acetylated as described, by using 6 μL of acetic anhydride per mg LDL protein. Excess reagents were removed by gel filtration as described above.

The degrees of acetylation and oxidation were assessed using nondenaturing agarose gel electrophoresis on 1% Universal agarose gels (Ciba-Corning) in Tris-barbitone buffer (pH 8.6) at 90 V for 45 minutes. The LDL band was visualized with fat red 7B stain. A relative electrophoretic mobility (REM) of 2 to 3, with native LDL as a reference (REM=1), was considered satisfactory.

Measurements of Lipoprotein Oxidation Products

Samples of cell lysates and LDL were extracted into n-hexane and analyzed by reverse-phase high-performance liquid chromatography as previously described. The formation of the oxidation products cholesteryl linoleate hydroperoxide, cholesteryl linoleate hydroxide, and 7-ketocholesterol, as well as consumption of cholesterol and individual cholesteryl esters, was measured to follow the progress of oxidation, as described previously.

Reagents

The following reagents were obtained from commercial sources: [³H]Tdr (80 Ci/mmol; Amersham Corp) and FCS (CSL). The following reagents were obtained as gifts: recombinant human CSF-1 (M-CSF), which is biologically cross-reactive on murine cells (Chiron Corp, Emeryville, Calif), and recombinant murine GM-CSF (DNAX, Palo Alto, Calif).

All other reagents were of analytical grade. All practical precautions for minimizing endotoxin contamination were taken. Solutions were made in pyrogen-free water (Delta West), and endotoxin levels were routinely monitored by Limulus lysate tests (CSL), with the minimum detectable level being 0.01 ng/mL.

Results

ox-LDL Induces BMM [³H]Tdr Incorporation

BMMs do not survive when deprived of CSF-1 and, as for other hemopoietic cells, death appears to be by apoptosis (Reference 29 and A. Jaworowski et al, unpublished observations, 1998). When ox-LDL (50 μg/mL) was added to CSF-1–starved BMMs, they remained attached to the tissue-culture plate (see below) and exhibited a granular appearance in the cytoplasm and enhanced lipid content when they were stained with Sudan red. In fact, as shown in Table 1, the cells incorporated [³H]Tdr, suggesting DNA synthesis (see below). In this particular experiment, the ox-LDL was present

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TABLE 1. Effect of Modified LDLs on BMM [3H]TdR Incorporation

<table>
<thead>
<tr>
<th>Addition</th>
<th>[3H]TdR Incorporation, cpm x 10^-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>2.9±0.3</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>92.6±4.8</td>
</tr>
<tr>
<td>Acetylated LDL</td>
<td>43.4±0.4</td>
</tr>
<tr>
<td>LDL</td>
<td>3.6±0.6</td>
</tr>
<tr>
<td>CSF-1</td>
<td>133.6±7.1</td>
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</tbody>
</table>

Quiescent BMM (see Methods) were treated with 50 μg/mL of Ox-LDL, acetylated LDL, or native LDL or with CSF-1 (5000 U/mL) as a positive control. After 24 hours, the cultures were pulsed with [3H]TdR and harvested at 72 hours. The data are expressed as mean±SEM from triplicate cultures.

Figure 1. BMM [3H]TdR incorporation after continuous exposure to increasing ox-LDL concentrations. Quiescent BMMs (i.e., CSF-1 removed for 24 hours) were treated with increasing concentrations of ox-LDL. After 24 hours, the triplicate cultures were pulsed with [3H]TdR and harvested at 72 hours. The data are expressed as fold stimulation (mean±SEM) relative to the [3H]TdR incorporation value for untreated cells (370±80 cpm). In the same experiment, the mean fold stimulation for cultures containing an optimal CSF-1 concentration (5000 U/mL) was 50±3. Error bars were omitted when they were smaller than the symbol.

Figure 2. Time course of BMM pretreatment with ox-LDL for subsequent [3H]TdR incorporation. a, Quiescent BMMs were treated with 50 μg/mL ox-LDL for different times; at each time point the ox-LDL was removed by washing the cells twice in PBS, followed by a further 48-hour incubation in culture medium containing [3H]TdR. The data are expressed as mean values ±SEM from triplicate cultures. b, As in a, but the pretreatment periods with ox-LDL before its removal were 8 and 24 hours, followed by the 48-hour incubation in the presence of [3H]TdR. For another group the ox-LDL was left in the cultures for 72 hours, with the [3H]TdR incorporation pulse over the last 48 hours. Error bars were omitted when they were smaller than the symbol.

During the 72-hour time period over which the [3H]TdR incorporation was measured. The Table also shows that acetylated LDL can also induce [3H]TdR incorporation, but native LDL failed to do so; the [3H]TdR incorporation due to an optimal CSF-1 concentration is included for comparison. The effect of the Cu²⁺ oxidation was not restricted to LDL, as ox-HDL (50 μg/mL) (but not HDL₃) was also active (data not shown).

The dose dependence for the ox-LDL effect over a 72-hour period is presented in Figure 1. In this representative experiment toxic effects were noted at 100 μg/mL, with cells detaching from the dish even more rapidly than did untreated cells. In the particular experiment whose data are presented in Figure 1, the fold stimulation at the optimal ox-LDL concentration was less than that observed at an optimal CSF-1 concentration (5000 U/mL); in some experiments with different ox-LDL preparations, but not usually, the ox-LDL effects were as high as the value obtained with this dose of CSF-1. Native LDL was inactive in similar experiments over the concentration range of 3 to 100 μg/mL (data not shown).

We next determined whether the ox-LDL needed to be present continuously for the above effects on BMM [3H]TdR incorporation to occur. In Figure 2a we demonstrate that a mere 2-hour exposure to ox-LDL (50 μg/mL), followed by its removal, is sufficient to induce [3H]TdR incorporation measured over a subsequent 48-hour period; in Figure 2b, it is shown that extending this pretreatment period to 24 hours in a separate experiment leads to a greater effect.

In Figure 3a, the effect of the degree of LDL oxidation, controlled in turn by the oxidation time, on the extent of [3H]TdR incorporation into BMMs is presented. This Figure shows that “active” ox-LDL was produced after an oxidation period of 2 to 4 hours, with the activity still increasing over the 24-hour time period examined. It should be noted that a 24-hour oxidation period was the one routinely used in our studies. A kinetic analysis of the changes in some LDL oxidation products is presented in Figure 3b.

When the kinetics of the [3H]TdR incorporation into quiescent BMMs due to ox-LDL (50 μg/mL) was followed, it was found that the first detectable changes were clearly observed between 12 and 22 hours, which is slightly later than those due to endogenous CSF-1 (5000 U/mL), the latter occurring between 10 and 12 hours (data not shown). However, it should be borne in mind, as mentioned above, that the degree of [3H]TdR incorporation due to ox-LDL is usually smaller than that resulting from CSF-1 action, which makes it more difficult to detect any earlier changes. Similar observations to those in CBA BMMs were found for BMMs from C57BL/6, MRL lpr/lpr, and MRL +/+ mice (data not shown).

Minimally modified ox-LDL can induce CSF-1 in cultured human aortic endothelial cells, L cells and human aortic smooth muscle cells, whereas it has been indicated that ox-LDL exerts its proliferative effect on murine peritoneal macrophages by production of endogenous GM-CSF.
test whether the effects of ox-LDL might be due to endogenous CSF-1 or GM-CSF production, we tested BMMs from mice deficient in both CSF-1 and GM-CSF, obtained by interbreeding op/op with GM-CSF\(^2/2\) mice.\(^33\) In the presence of 50 \(\mu\)g/mL ox-LDL, BMMs from these mice showed similar morphological changes, lipid uptake, enhanced survival, and enhancement of \([3H]Tdr\) incorporation in the 4 experiments performed; ie, their response was similar to those of CBA BMMs. Data for \([3H]Tdr\) incorporation from a representative experiment are presented in Table 2. Also, 48-hour supernatants from CBA BMM pretreatment with ox-LDL (50 \(\mu\)g/mL) failed to stimulate BMM \([3H]Tdr\) incorporation in different culture wells. From these data it would appear that neither endogenous CSF-1 nor GM-CSF, or perhaps even another cytokine, is necessary for the observed effects of ox-LDL.

Effects of ox-LDL in the Presence of CSF-1
CSF-1 circulates at a low concentration, which is believed to contribute to monocyte/macrophage survival\(^34\); in other words, it is likely that monocytes/macrophages are usually exposed to such a low CSF-1 concentration in vivo. We next measured the \([3H]Tdr\) incorporation into BMMs in the presence of low CSF-1 concentrations, which help to maintain survival and induce minimal DNA synthesis, and in the presence of higher mitogenic concentrations.\(^35\) Two sets of culture conditions were used, viz, in which the cells were either deprived of CSF-1 for 24 hours before ox-LDL treatment, as in the experiments performed above (quiescent BMMs), or in which ox-LDL was added at the time of CSF-1 removal from the cycling cells. Under both conditions (Figure 4a and 4b, respectively), ox-LDL treatment resulted in a dramatic, synergistic effect at low CSF-1 concentrations. At high CSF-1 concentrations some reduction in DNA synthesis in the presence of ox-LDL was often but not always observed; for some reason this reduction was more noticeable within the first 24 hours after CSF-1 and ox-LDL addition than at later

<p>| TABLE 2. Ox-LDL and op/op GM-CSF(^{-/-}) BMM DNA Synthesis |
|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Addition</th>
<th>([3H]Tdr) Incorporation, cpm.(\times)10(^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quiescent BMM</td>
<td>2.1±0.3</td>
</tr>
<tr>
<td>0x-LDL</td>
<td>64.0±2.6</td>
</tr>
<tr>
<td>CSF-1</td>
<td>80.4±7.8</td>
</tr>
</tbody>
</table>

Quiescent BMM from op/op GM-CSF\(^{-/-}\) mice\(^33\) were treated with ox-LDL (50 \(\mu\)g/mL) or CSF-1 (5000 U/mL). As in Table 1, cultures were pulsed after 24 hours with \([3H]Tdr\) and harvested at 72 hours and the data are expressed as mean±SEM from triplicate cultures.

Figure 3. Degree of oxidation of LDL and BMM \([3H]Tdr\) incorporation. a, Quiescent BMMs were treated for 24 hours with LDL (50 \(\mu\)g/mL) that had been oxidized for different times. At this time, the cells were washed twice in PBS, and medium was added again with \([3H]Tdr\) for a further 48 hours. The data are expressed as mean values ±SEM from triplicate cultures. Error bars were omitted when they were smaller than the symbol. b, Samples of each LDL at each oxidation time were also taken to assess the degree of oxidation. Cholesterol linoleyl hydroperoxide (●), cholesterol linoleyl hydroxide (▲), and 7-ketocholesterol (Œ) were measured by reverse-phase high-performance liquid chromatography. Data are mean±SD of triplicate determinations. REM (□) was measured by agarose electrophoresis, with native LDL as a reference (see Methods).

Figure 4. Effect of ox-LDL on BMM \([3H]Tdr\) incorporation in the presence of CSF-1 a, quiescent BMMs (ie, CSF-1 removed for 24 hours) were either left untreated (●) or treated with ox-LDL (50 \(\mu\)g/mL) (▲) for 24 hours. At this time, the cells were washed twice in PBS, and different concentrations of CSF-1 were added twice daily for the 48-hour period; the cells were then pulsed with \([3H]Tdr\) for the final 24 hours. In b, BMMs, from which CSF-1 had just been removed, were either left untreated (●) or treated with ox-LDL (50 \(\mu\)g/mL) (▲) for 24 hours; ie, the CSF-1 used for BMM growth was removed just before the ox-LDL addition. The rest of the protocol was as in a. The data are expressed as mean values ±SEM from triplicate cultures. Error bars were omitted when they were smaller than the symbol.
leled the relative degree of [3H]TdR incorporation. It was also changes in the proportion of cells with labeled nuclei paral-
tively. When all of the treatments were considered, the
for the experiment in Figure 4b were 55% and 2%, respec-
tively. When all of the treatments were considered, the
46% of the ox-LDL–treated BMMs had labeled nuclei com-
pared with none in the untreated cells; the corresponding data
were harvested, stained with PI, and analyzed for DNA content
by using a flow cytometer (see Methods).

To confirm whether the increase in [3H]TdR incorporation
was reduced from 30% to 15% by the prior addition of
ox-LDL (50 μg/mL), indicating increased cell survival.
Measurement of apoptosis by DNA laddering and terminal
deoxyribonucleotidyl transferase–mediated nick end labeling
showed that ox-LDL, like CSF-1, reduced apoptosis in
ox-LDL–treated quiescent BMMs was still 8×10^4 to undetectable over the
following 72 hours; in contrast, the number of viable ox-
drooped dramatically, from 8×10^4 to 5×10^3, with ox-LDL treatment maintaining substantially the cell
number (1.1×10^5; Figure 6b). Under both culture conditions,
ox-LDL synergized with suboptimal CSF-1 concentrations,
leading to an increase in cell number rather than mere
maintenance. Again the degree of LDL oxidation was signif-
icant, as LDL that had been oxidized for only 4 hours was
significant, as LDL that had been oxidized for only 4 hours was
enhanced the proportion of cells in the S phase due to
low-dose CSF-1 (160 U/mL) (Figure 5d versus Figure 5c).
The combination of ox-LDL and low-dose CSF-1 gave a
similar percentage of S-phase cells as an optimal CSF-1
concentration (5000 U/mL; Figure 5b). These findings sug-
gest that the [3H]TdR incorporation resulting from ox-LDL
treatment was due to enhanced DNA synthesis and not from
DNA repair as a result of damage.

A more detailed analysis of the sub-G0/G1 DNA content in
quiescent BMMs as a measure of cell viability was carried out
by this methodology. Typically the proportion of cells after a
further 24 hours in culture in this region of low DNA content
time points. In separate experiments when different doses of
ox-LDL were used in the presence of a single CSF-1
concentration (320 U/mL), the ox-LDL was effective at a
concentration as low as 3 μg/mL. As for cultures in the
absence of added CSF-1, an oxidation period of between 2
and 4 hours was required for the ox-LDL to be again effective
in the presence of a suboptimal CSF-1 concentration. Acety-
lated LDL and ox-HDL2 were also able to give a higher
[3H]TdR incorporation in the presence of low CSF-1 con-
centrations (160 to 320 U/mL) than in the absence of the growth
factor (data not shown).

In the same experiments whose data are presented in
Figure 4a and 4b, the proportion of cells that had incorporated
[3H]TdR into the nucleus was monitored by autoradiography.
For the experiment whose data are provided in Figure 4a,
46% of the ox-LDL–treated BMMs had labeled nuclei com-
pared with none in the untreated cells; the corresponding data
for the experiment in Figure 4b were 55% and 2%, respec-
tively. When all of the treatments were considered, the
changes in the proportion of cells with labeled nuclei paral-
leled the relative degree of [3H]TdR incorporation. It was also noted that the number of grains over the nuclei were similar in the
ox-LDL– and CSF-1–treated groups (data not shown).
To confirm whether the increase in [3H]TdR incorporation
observed above was due to DNA synthesis rather than DNA
repair, we measured DNA content by PI staining and flow
cytometry. As shown in Figure 5, ox-LDL treatment (Figure 5c)
increased the proportion of cells in the S phase at 24 hours
compared with untreated cells (Figure 5a); in addition,
ox-LDL enhanced the proportion of cells in the S phase due to
low-dose CSF-1 (160 U/mL) (Figure 5e versus Figure 5d).
Figure 5. Effect of ox-LDL on BMM DNA content. BMMs, from
which CSF-1 had just been removed (see Figure 4b), were incu-
bated in a, RPMI and 10% FCS alone or together with b, CSF-1
(5000 U/mL); c, ox-LDL (50 μg/mL); d, CSF-1 (160 U/mL); or e,
CSF-1 (160 U/mL)+ox-LDL (50 μg/mL) for 24 hours. The cells
were harvested, stained with PI, and analyzed for DNA content
by using a flow cytometer (see Methods).

Effects of ox-LDL on BMM Cell Numbers
We next monitored cell number to ascertain whether in-
creases in BMM number could actually be occurring under
any of the culture conditions used in Figure 4a and 4b. The
corresponding cell number data are presented in Figure 6a
and 6b, respectively. Figure 6a shows that for quiescent
BMMs in the absence of CSF-1, the viable cell number
dropped dramatically, from 8×10^4 to undetectable over the
following 72 hours; in contrast, the number of viable ox-
LDL–treated quiescent BMMs was still 8×10^4 at the end of the
experiment. Likewise, for BMMs from which CSF-1 had
just been removed, the viable cell number again was lowered
dramatically over the next 72 hours, from 1.4×10^5 to 5×10^3,
with ox-LDL treatment maintaining substantially the cell
number (1.1×10^5; Figure 6b). Under both culture conditions,
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maintenance. Again the degree of LDL oxidation was signif-
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again less effective (data not shown). In other experiments,
ox-LDL by itself, even though it was able to retard the loss of
viable cells, was not able to maintain cell number, particu-

Figure 6. Effect of ox-LDL on BMM cell number in the absence
and presence of CSF-1. These data are from the same experi-
ment whose data are presented in Figure 4a and 4b, except
that cell number and not [3H]TdR incorporation was monitored.
In a, the number of quiescent BMMs (ie, CSF-1 removed for 24
hours) at the start of the experiment was 8×10^4 (indicated by
the dotted line and t=0). These BMMs were then either left
untreated (○●) or treated with ox-LDL (50 μg/mL □△); at this
time, different concentrations of CSF-1 were added twice daily,
and viable cells were counted after 72 hours by hemocytometer
(see Methods). In b, BMMs (1.4×10^5 cells; again indicated by
the dotted line and t=0), from which CSF-1 had just been
removed, were either left untreated (●●) or treated with ox-LDL
(60 μg/mL □△); ie, the CSF-1 used for BMM growth was
removed just before the ox-LDL addition. The rest of the proto-
ocol was as in a. The data are expressed as mean values ± SEM
from triplicate cultures. Error bars were omitted when they were
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Figure 5. Effect of ox-LDL on BMM DNA content. BMMs, from
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were harvested, stained with PI, and analyzed for DNA content
by using a flow cytometer (see Methods).
larly when the starting BMM population was “quiescent” (data not shown).

Dimethylthiazolyl diphenyltetrazolium bromide (MTT) activity can be used to quantify cell number, including BMMs treated with CSF-1, and this technique was used to follow cell number changes with ox-LDL–treated murine peritoneal macrophages. However, we found that the MTT activity of quiescent BMMs could be elevated 3- to 8-fold after overnight treatment with ox-LDL (50 μg/mL). This dramatic change presumably represents enhanced mitochondrial activity and illustrates that this method cannot be used to measure cell number in ox-LDL–treated BMM cultures and possibly, for other ox-LDL–treated macrophage populations.

**Effect of ox-LDL in the Presence of GM-CSF**

The BMMs that are derived from progenitors in response to CSF-1 elicit a poor proliferative response to GM-CSF compared with that in response to CSF-1. However, we show in Figure 7 that, when BMM DNA synthesis is monitored in the presence of varying concentrations of ox-LDL and an optimal GM-CSF concentration (1000 U/mL), there is a synergistic effect.

**Discussion**

In this study we have demonstrated that treatment of BMMs with ox-LDL, but not with native LDL, leads to survival and DNA synthesis, as well as to an enhanced response to the proliferative actions of CSF-1 and GM-CSF; for CSF-1 the effect was noticeable at low and suboptimal doses of the growth factor. The effects of ox-LDL by itself occurred at low doses, were dependent on the degree of oxidation, were found in preloaded cells, and even occurred in the absence of endogenous CSF-1 or GM-CSF; our data would seem to be different from those reported for the proliferative effect of ox-LDL on murine peritoneal macrophages, which has been indicated to be due to the production of GM-CSF. Monocyte adhesion and infiltration of arterial tissue are 2 of the earliest events in human and experimental atherogenesis, and cytokine production by them or by foam cells may be important in the initiation and amplification of inflammation in the atheroma. The importance of CSF-1 as a maintenance factor for monocytes/macrophages has led to the speculation that it may be required to maintain long-term survival of macrophages in lesions. Even though care must be exercised in extrapolating from these in vitro studies, we suggest that low doses of ox-LDL, and perhaps other modified forms, could maintain macrophage (and foam cell) survival and therefore lengthen their tenure in a lesion by suppressing the apoptotic process with possible consequences for plaque progression. Others have found that ox-LDL (≥100 μg/mL) causes apoptotic death in monocytes/macrophages in vitro, and macrophage (foam cell) death has been proposed to contribute to the lipid core of the atheroma. We found that such high ox-LDL concentrations were also toxic to BMMs. Perhaps in vivo the macrophage response may also depend on the dose of modified LDL, with lower doses contributing to cell survival and higher doses leading to cell death.

As mentioned previously, there is evidence in both animal and human atherosclerotic lesions that macrophage-derived foam cells represent a significant proportion of the cycling cells. Very recently, Rekhter and Gordon reported that in human carotid plaques the preponderant proliferative cell type in the intima was the monocyte/macrophage and that foam cell–rich regions mostly displayed proliferation among the macrophages. Even though the level of proliferation is low in vivo, such a low level occurring over many years, with associated other tissue changes, could produce an occlusive arterial mass by midlife. It has been reported that murine peritoneal macrophages proliferate in response to ox-LDL (see below). We used BMMs as a model because, like macrophage-derived foam cells, they depend on CSF-1 for their development; they are also relatively homogeneous and are well studied for their survival and mitogenic responses to CSF-1 and GM-CSF. Our findings indicate that ox-LDL can induce DNA synthesis in BMMs. The number of divisions that ox-LDL–loaded BMMs can undergo is unknown, and presumably the number of cells present in the ox-LDL–treated cultures is a balance between those surviving, proliferating, and dying. It should be noted that the MTT assay has been used to monitor increased murine peritoneal macrophage cell numbers after modified LDL treatment. We found above that this assay could not be used to quantify numbers of ox-LDL–treated BMMs since MTT activity was altered drastically on ox-LDL loading. Our results suggest that caution be exercised when using this assay to quantify numbers of ox-LDL–treated macrophage populations.

As mentioned, it has been proposed that CSF-1 may have a role in maintaining long-term survival and proliferation of macrophages (foam cells) in atherosclerotic lesions. This factor also circulates at low concentrations that maintain monocyte/macrophage survival. It is possible that there may be higher local levels in plaques, although such levels have not been accurately measured until now. Our results indicate that macrophages, when “loaded” with ox-LDL, are “primed” so that they are able to proliferate better in the presence of CSF-1 doses that are suboptimal, including “survival” doses, and that may be similar to those found in atheromas. The biochemical mechanisms underlying this phenomenon, as well as the clinical relevance, await clarification.
GM-CSF expression is associated with macrophage proliferation in rabbit atheromatous lesions,\textsuperscript{20} it can be produced in vitro by vascular endothelial and smooth muscle cells,\textsuperscript{8–11} and it is found at other sites of inflammation.\textsuperscript{42} GM-CSF has been implicated in inflammatory diseases by virtue of its actions on monocytes/macrophages and neutrophils and, like CSF-1, can induce the proliferation of a subpopulation of human peripheral blood monocytes.\textsuperscript{43} We also showed above that the ox-LDL–loaded macrophages underwent enhanced DNA synthesis in response to this growth factor. The significance of this observation also awaits analysis.

It is possible that the ox-LDL effects reported above are at least partially dependent on the concomitant increased cellular content of cholesterol, because ox-LDL is a scavenger-receptor ligand\textsuperscript{44} and stimulates cholesterol loading when incubated with macrophages.\textsuperscript{45} It can be seen that acetylated LDL, which also has these properties,\textsuperscript{44,45} also stimulates the receptor ligand\textsuperscript{44} and stimulates cholesterol loading when incubated with macrophages.\textsuperscript{45} It can be seen that acetylated LDL, which also has these properties,\textsuperscript{44,45} also stimulates scavenger receptor expression and function. and -independent pathways in rabbits.\textsuperscript{1} It is therefore possible that such molecules may stimulate macrophage DNA synthesis in vitro and in atherosclerotic plaques. Another possibility is that lysophosphatidylcholine in ox-LDL is also contributing.\textsuperscript{32}

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