Comparison of macrophage responses to oxidized low-density lipoprotein and macrophage colony-stimulating factor (M-CSF or CSF-1)

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Modification of low-density lipoprotein (LDL), for example by oxidation, could be involved in foam cell formation and proliferation observed in atherosclerotic lesions. Macrophage colony-stimulating factor (CSF-1 or M-CSF) has been implicated in foam cell development. It has been reported previously that oxidized LDL (ox.LDL) and CSF-1 synergistically stimulate DNA synthesis in murine bone-marrow-derived macrophages (BMM). The critical signal-transduction cascades responsible for the proliferative response to ox.LDL, as well as their relationship to those mediating CSF-1 action, are unknown. We report here that ox.LDL stimulated extracellular signal-regulated protein kinase (ERK)-1, ERK-2 and phosphoinositide 3-kinase activities in BMM but to a weaker extent than optimal CSF-1 concentrations at the time points examined. Inhibitor studies suggested at least a partial role for these kinases, as well as p70 S6-kinase, in ox.LDL-induced macrophage survival and DNA synthesis. For the DNA synthesis response to CSF-1, the degree of inhibition by PD98059, wortmannin and rapamycin was significant at low CSF-1 concentrations but was reduced as the CSF-1 dose increased. Using BMM from CSF-1-deficient mice (op/op) and a neutralizing antibody approach, we found no evidence for an essential role for endogenous CSF-1 in ox.LDL-mediated survival or DNA synthesis; likewise, with the same approaches, no evidence was obtained for an essential role for endogenous granulocyte/macrophage-CSF in ox.LDL-mediated macrophage survival and, in contrast with the literature, ox.LDL-induced macrophage DNA synthesis.

Key words: DNA synthesis, ERK, foam cell, macrophage survival, phosphoinositide 3-kinase.

INTRODUCTION

Cholesterol ester-filled macrophages or 'foam' cells are an early and prominent feature of atherosclerotic lesions [1]. Evidence suggests that low-density lipoprotein (LDL), the major cholesterol-carrying lipoprotein in blood, when modified in some way, can become atherogenic. LDL oxidation can occur *in vivo* and oxidized LDL (ox.LDL) is often considered to be atherogenic since, for example, it can cause the formation of macrophage-derived foam cells *in vitro* [2]. One of the features of atherosclerotic lesions in arterial walls is cellular hyperplasia. Reports of both human and animal studies suggest that the macrophage-derived foam cells are making a significant contribution to the cellular proliferation observed, particularly in the early stages (see [3–8]).

It has been reported that ox.LDL is mitogenic for murine peritoneal macrophages [9–12] and human macrophages [13]. We have recently found that the survival of an *in vitro*-derived murine macrophage population, namely bone-marrow-derived macrophages (BMM), was maintained by ox.LDL [14]; also the BMM could proliferate in response to ox.LDL, particularly in the presence of low concentrations of macrophage colony-stimulating factor (CSF-1 or M-CSF). Since CSF-1 normally circulates at such low concentrations [15], it was proposed in that study that this observation is likely to have relevance *in vivo*. In addition to showing enhanced survival, both ox.LDL- and CSF-1-treated BMM spread and attach to a tissue-culture surface better than untreated controls [14]. The degree of stimulation of BMM DNA synthesis by ox.LDL was usually not quite as marked as that found with optimal CSF-1 concentrations but there was a dramatic synergistic effect with sub-optimal CSF-1 doses [14]. In BMM, the signalling responses to CSF-1 have been well studied, including a number of dose-dependent early biochemical responses [16], such as enhanced phosphoinositide 3-kinase (PI 3-kinase) and extracellular signal-regulated protein kinase (ERK) activities [17–19]. These two responses have been widely implicated in growth-factor-mediated cell survival and proliferation, including in CSF-1-treated monocytes for the PI 3-kinase activity [20,21]. We therefore chose to determine whether ox.LDL activated these pathways as CSF-1 does and to determine their significance for the responses of macrophages to ox.LDL.

Prior to initiating any analysis of the signal-transduction cascades relevant to the ox.LDL-mediated induction of macrophage survival and DNA synthesis, it is important that the contribution of any endogenously produced CSF-1 or granulocyte/macrophage CSF (GM-CSF) be determined. If such an indirect mechanism were significant, then signalling pathways governing CSF formation and/or action may be studied inadvertently instead of those relevant to a direct effect of ox.LDL on macrophage survival and proliferation. CSF-1 and its receptor have been detected in atherosclerotic plaques [22,23]. These data and others have led to the suggestion that CSF-1 in the plaque microenvironment could promote the recruitment and retention of mononuclear phagocytes and subsequent foam cell formation [22–28]. Minimally modified ox.LDL can induce CSF-1 in vascular cells *in vitro* [24,29] and endogenous CSF-1 can influence monocyte/macrophage functions *in vitro* [30]. Therefore endogenous CSF-1 in ox.LDL-treated macrophage cultures could

Abbreviations used: LDL, low-density lipoprotein; ox.LDL, oxidized LDL; GM-CSF, granulocyte/macrophage colony-stimulating factor; CSF-1, macrophage CSF; BMM, bone-marrow-derived macrophages; RPM, resident peritoneal macrophages; ^{3}H-TdR, [*methyl*-^{3}H]thymidine; FBS, heat-activated fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; PI 3-kinase, phosphoinositide 3-kinase; ERK, extracellular signal-regulated protein kinase; MEK, mitogen-activated protein kinase/ERK kinase.

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be responsible for the enhanced survival and induction of DNA synthesis. It has been reported recently that GM-CSF plays an essential role in ox.LDL-induced macrophage DNA synthesis [11,31,32] and that glucocorticoids inhibit such growth by suppressing GM-CSF expression [33].

In the present study, BMM were usually studied since they have been widely used to explore CSF-1-dependent signal-transduction pathways relevant to enhanced survival and proliferation [16–19,34–37]; unlike peritoneal macrophages, they can be obtained readily in large numbers, they all depend on CSF-1 for their survival and they commence DNA synthesis within 10–12 h of CSF-1 addition [38]. The last property is particularly convenient for the analysis of the specific effects of potentially growth-inhibitory drugs because the chances of long-term toxic (i.e. non-specific) effects are reduced.

Using BMM from CSF-deficient mice or blocking antibodies, no evidence could be found in this report for an essential role for either CSF-1 or GM-CSF in ox.LDL-induced macrophage survival and DNA synthesis. ox.LDL induced small increases in ERK and PI 3-kinase activities compared with optimal CSF-1 concentrations. Studies using inhibitors indicated that the ox.LDL-induced macrophage survival and/or DNA synthesis are partially dependent on ERK, PI 3-kinase and p70 S6-kinase activities.

EXPERIMENTAL

Mice

The following mice were used at 8–12 weeks of age: male CBA mice (used generally), and male GM-CSF$^{-/-}$ and wild-type controls (GM-CSF$^{+/+}$) (Ludwig Institute for Cancer Research, Parkville, VIC, Australia) [39,40]. Osteopetrotic (op/op) mutant mice [41] and their littermate wild-type controls, each on the C57BL/6 and C3HeB/FeJ background, were obtained from the Ludwig Institute and maintained in micro-isolators within its animal house facility. Following weaning, the op/op mice, which lack incisors, were maintained on a diet containing equal amounts of powdered chow and Ensure nutritional powder (VHA Trading Co., Mulgrave, Australia), mixed in sterile water.

BMM

BMM were generated as adherent cells from their non-adherent progenitors in bone marrow as described before [16,34]. They were grown to confluence in 24-well tissue-culture plates (Nunc, Naperville, IL, U.S.A.) for 5–6 days in RPMI 1640 medium supplemented with 20 mM Hepes, 15 % heat-inactivated fetal bovine serum (FBS) and 30 % L cell-conditioned medium (as a crude source of CSF-1). For the preparation of the CSF-deficient BMM and their wild-type controls, a slightly modified procedure was adopted to ensure comparable initial cell numbers [42]. For this procedure, non-adherent precursors, generated after 3 days from CSF-1-treated bone-marrow cells in RPMI 1640/10 % FBS, were plated into non-tissue-culture plastic dishes (10 cm diameter; Disposable Products, Adelaide, South Australia, Australia) at 1.2×10^6 cells/dish. After a further 3 days of culture in CSF-1-containing medium (see above), the adherent BMM were aspirated from the surface, plated at 7.5×10^4 or 10^5 cells/well in 24-well tissue-culture plates in the same medium, and allowed to adhere for another 24 h.

The BMM are a relatively pure and homogeneous population with $\geqslant 95$ % of the adherent cells binding CSF-1 [34,38]. Cells derived by the two procedures were prepared for experiments by washing twice with PBS, and recultured in growth medium without L cell-conditioned medium. BMM at this stage were usually 'starved' of growth factor for 24 h before use to render the cells quiescent [34,38].

Resident peritoneal macrophages (RPM)

Peritoneal cells were washed from the unstimulated peritoneal cavities of mice by lavage with 5 ml of ice-cold sterile PBS [43]. For the generation of the RPM cultures, the peritoneal cells were plated at 4×10^5 cells/ml of RPMI 1640/5 % FBS in 24-well plates and allowed to adhere for 18 h at 37 °C. Cultures were washed twice with PBS and cultured in RPMI 1640/5 % FBS in the presence of various agents.

DNA synthesis

DNA synthesis was measured as the incorporation of [*methyl*-^{3}H]thymidine (^{3}H-TdR; 2 μCi/ml) [34,42]. Uptake was stopped by removal of the culture medium and solubilization of the cells in 0.2 M NaOH. The trichloroacetic acid-precipitable material was recovered using an Inotech cell harvester (Berthold-Australia, Bundooma, Northern Territory, Australia). The incorporation of label was estimated using scintillation spectroscopy.

Cell numbers

For the quantification of the number of macrophages in cultures, the medium was removed, and the cells gently scraped. Viable cells were counted in a haemocytometer using Trypan Blue exclusion. The proportion of viable cells was confirmed by flow cytometry by propidium iodide staining using a FACS Caliber flow cytometer.

ERK activity

Following treatment, BMM were washed in PBS, lysed and Western-blotted with phospho-ERK-1 and -ERK-2 antibodies as described previously [19,44].

PI 3-kinase activity

Following treatment, cells were washed in PBS and then solubilized in lysis buffer containing 1 % Triton X-100 and then 1.0 mg of extract was immunoprecipitated with anti-phosphotyrosine antibody (4G10), as reported previously [18]. The immunoprecipitates were washed and assayed for PI-3 kinase activity *in vitro* as described in [17].

LDL preparation

As described in [14,45], human LDL was isolated from healthy fasting volunteers in the presence of 3 mM EDTA by discontinuous density-gradient ultracentrifugation in the density range $\rho = 1.02$–1.05. The isolated LDL was sterilized by membrane filtration (0.45 μm), and stored in the dark at 4 °C under N_2. LDL preparations were used within 1 week of isolation. All materials and solutions were pre-treated to remove endotoxin.

ox.LDL preparation

For the preparation of ox.LDL, LDL was desalted into PBS by two passages over Sephadex G-25 (PD-10; Pharmacia) in series to remove KBr and EDTA [14,45]. Copper oxidation was achieved by incubating LDL (1 mg of protein/ml) in PBS with a sterile solution of $CuC1_2$ (final concentration 20 μM) at 37 °C for 24 h [14,45]. Oxidation was arrested by addition of serum or by dialysis against PBS to remove excess $CuCl_2$, and the material was used within 7–14 days of preparation.

The degree of oxidation was assessed using non-denaturing agarose-gel electrophoresis on 1 % Universal Agarose gels (Ciba-Corning) in Tris/barbitone buffer (pH 8.6) at 90 V for 45 min. The LDL band was visualized with Fast Red 7B stain. A relative electrophoretic mobility of 2–3, using native LDL as a reference, was considered satisfactory. LDL prepared by this method contained 225 ± 40 nmol of 7-oxocholesterol/mg of LDL protein. No peroxides were detectable at this stage of oxidation, being converted to more advanced oxidation products [45,46].

Reagents

The following reagents were obtained commercially: ^{3}H-TdR (80 Ci/mmol; Amersham Corp., Sydney, NSW, Australia); FBS (CSL, Parkville, Australia); PD98059 and anti-phospho-ERK antibodies (New England Biolabs, Beverly, MA, U.S.A.); wortmannin (Sapphire BioScientific, Alexandria, Australia); rapamycin (Sigma); anti-phosphotyrosine antibody (4G10; Upstate Biotechnology, Lake Placid, NY, U.S.A.).

The following reagents were obtained as gifts: recombinant human CSF-1 (M-CSF), which is biologically cross-reactive on murine cells (Chiron Corporation, Emeryville, CA, U.S.A.); recombinant murine GM-CSF (specific activity, 2 units/mg; Novartis, Vienna, Austria); purified monoclonal antibody (22E9) to GM-CSF and purified isotype (IgG2a) control, monoclonal antibody to β-galactosidase (J. Abrams, DNAX, Palo Alto, CA, U.S.A.); hybridoma (AFS-98), a source of a blocking antibody to murine CSF-1 receptor [47] (S.-I. Nishikawa, Kyoto, Japan); LY294002 (Eli Lilly, Indianapolis, IN, U.S.A.).

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, Bentley, Australia) and endotoxin levels were routinely monitored by limulus lysate tests, with the minimum detectable level being 0.01 ng/ml [34].

RESULTS

ox.LDL-induced macrophage DNA synthesis is independent of endogenous CSF-1

BMM require CSF-1 for their survival and, at higher CSF-1 doses, undergo DNA synthesis within 10–12 h [34,38]. They are therefore a suitable macrophage population to examine the effect of ox.LDL on both of these parameters. We showed previously that ox.LDL, but not native LDL, can promote BMM survival and also DNA synthesis, the latter response was generally weaker than the one with high CSF-1 concentrations [14]. We attempted to monitor viable cell numbers by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] formazan levels, as has been done in other studies monitoring macrophage proliferation to ox.LDL [10–12,48]. However, we found that MTT formazan levels could not be used to monitor changes in BMM viable cell numbers due to ox.LDL, since addition of ox.LDL to BMM caused a dramatic enhancement of the MTT formazan levels even after a 24 h incubation (> 3-fold), which was obviously not reflected in an increase in cell number over this short time period [38] (results not shown). Similar findings were made with murine RPM. CSF-1 did not cause this dramatic increase in MTT formazan levels in either macrophage population over this period, highlighting a difference in the response of macrophages to the two stimuli.

Since we had observed previously [14] that the degree of DNA synthesis induced by ox.LDL is usually smaller than that seen with optimal CSF-1 and, since a synergistic effect with sub-optimal CSF-1 concentrations was noted, it was considered of interest to compare the respective signal-transduction cascades activated by the two stimuli. Prior to initiation of such studies it was deemed important to assess the possible involvement of endogenous CSF-1, particularly since minimally modified ox.LDL can induce CSF-1 in aortic endothelial and L cells, as well as in aortic smooth-muscle cells [24,29]. We have shown before, using BMM from mice deficient in both CSF-1 and GM-CSF activities, that neither of these CSFs was required for ox.LDL-induced DNA synthesis [14]. However, in that type of experiment the contribution of each CSF could not be assessed and a comparison with wild-type mice was not made. Using BMM from op/op (CSF-1-deficient) [41] and littermate-control mice expressing an active CSF-1, we demonstrate in the two cell populations that ox.LDL can equally prevent the loss in viable cell number upon CSF-1 withdrawal from the cultures (Figure 1a) and also induce DNA synthesis to a similar extent (Figure 1b). We showed before that ox.LDL prevented the loss in CBA-strain BMM cell death which occurs by apoptosis once the CSF-1 used to grow the cells is removed [14]. As an additional

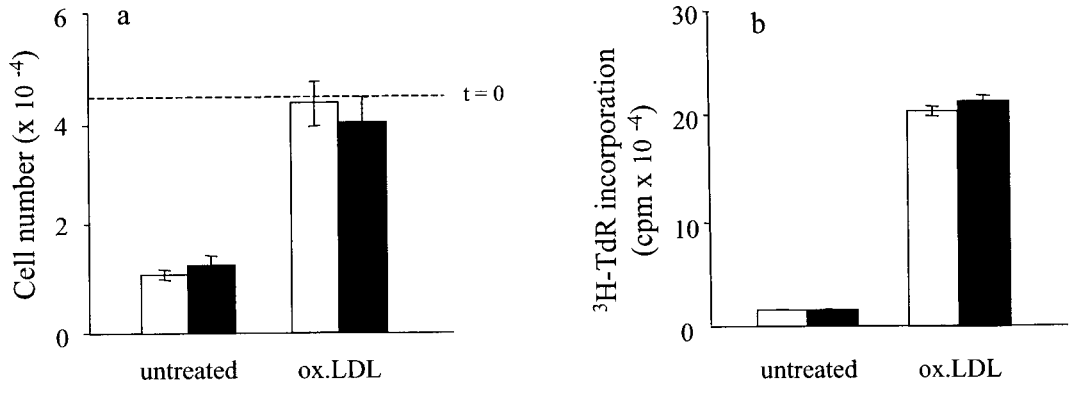

Figure 1 ox.LDL promotes survival and DNA synthesis in op/op BMM

Equal numbers of BMM from op/op and littermate-control mice were plated in CSF-1 overnight (see the Experimental section); at this time the cells were washed twice with PBS and were either untreated or treated with ox.LDL (50 μg of protein/ml). (**a**) Viable cell numbers were measured at this time ($t = 0$; dotted line) and 72 h later. (**b**) DNA synthesis was measured by ^{3}H-TdR incorporation between 24 and 72 h. Open bars, op/op; solid bars, op/op control littermates. Data are means ± S.E.M. from triplicate cultures from a representative experiment that was repeated twice more.

Table 1 Lack of effect of anti-CSF-1 receptor antibody on ox.LDL-induced BMM and RPM DNA synthesis

For the ox.LDL treatment, BMM from CBA mice were deprived of CSF-1 for 24 h (see the Experimental section) and CBA-strain RPM were prepared by plating 4×10^5 peritoneal cells from unstimulated peritoneal cavities and allowing them to adhere for 18 h at 37 °C (see the Experimental section). Both macrophage populations were left untreated or treated with ox.LDL (50 μg of protein/ml), in the absence or presence of a purified monoclonal antibody to the CSF-1 receptor (see the Experimental section). After 24 h, the cultures were washed twice with PBS and treated with medium (RPMI 1640/10% FBS), in the absence or presence of the antibody. DNA synthesis was monitored by pulsing at this time with ^{3}H-TdR for 48 h (BMM) or for 5 days (RPM). Data are means ± S.E.M. from triplicate cultures from representative experiments, which were repeated three (BMM) or four (RPM) times. In order to show the activity of the anti-CSF-1-receptor antibody, the CSF-1-deprived BMM and the RPM were also treated instead with CSF-1 (5000 units/ml), in the absence or presence of the antibody. DNA synthesis was monitored by pulsing at this time with ^{3}H-TdR for 24 h (BMM) or for 5 days (RPM). Data are means ± S.E.M. from triplicate cultures from representative experiments repeated three (BMM) or four (RPM) times.

	^{3}H-TdR incorporation (c.p.m. $\times 10^{-4}$)	
Treatment	BMM	RPM
No addition	0.9 ± 0.08	0.24 ± 0.01
ox.LDL	25.0 ± 0.6	1.2 ± 0.1
ox.LDL + anti-CSF-1 receptor	25.8 ± 0.3*	1.0 ± 0.1*
CSF-1	55.4 ± 1.6	6.7 ± 0.2
CSF-1 + anti-CSF-1 receptor	3.3 ± 0.1	0.21 ± 0.02

* Not significant compared with ox.LDL-treated group ($P > 0.05$).

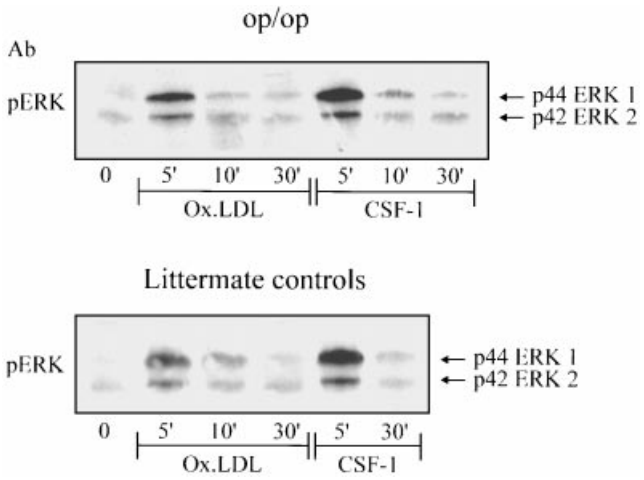

Figure 2 ox.LDL stimulates ERK activities in op/op BMM

BMM from both op/op and littermate-control mice were grown to confluence in 10 cm-diameter tissue-culture dishes and growth-arrested for 18 h (see the Experimental section). They were then treated with ox.LDL (50 μg of protein/ml) or CSF-1 (5000 units/ml) for the indicated times (5, 10 and 30 min). ERK-1 and ERK-2 activities were monitored in equal amounts of cell lysates by Western blotting using phospho-ERK antibody (pERK; see the Experimental section). Data are from a representative experiment that was repeated twice more.

approach, antibody to the CSF-1 receptor did not suppress the induction by ox.LDL of CBA-strain BMM or RPM DNA synthesis even though it blocked the corresponding effect of CSF-1 (Table 1). The above results indicate that endogenous CSF-1 is neither essential nor appearing to be making a significant contribution to the promotion of macrophage survival and DNA synthesis by ox.LDL. For both BMM, native LDL was once again inactive [14].

RPM are most often used to study ox.LDL-induced macrophage proliferation [9–12,31–33]. Some idea of the extent of the relative proliferative responses of BMM and RPM to CSF-1 (and to ox.LDL) can be obtained from Table 1. The ^{3}H-TdR incorporation in the CSF-1-treated BMM is far higher than the value for similar numbers of CSF-1-treated RPM in spite of the ^{3}H-TdR pulse being over 5 days for the latter population and over only 1 day for the former.

ox.LDL stimulates BMM ERK activity

Increased ERK activity is often correlated with enhanced cell survival and proliferation [20]; we have reported before that CSF-1 elevates ERK-1 and ERK-2 activities in BMM [19,35,44]. We see in Figure 2 that ox.LDL stimulates ERK-1 and ERK-2 activities both in op/op BMM and in BMM from littermate controls; a comparison with the effect of an optimal concentration of CSF-1 is presented and it can be observed that the degree of ox.LDL-induced stimulation was less at the time points examined. This result illustrates the potential usefulness of macrophages from op/op mice to study ox.LDL-induced signalling events without the complication of a contribution from any endogenous CSF-1 synthesized by the cells.

We have published that suppression of BMM apoptosis by high concentrations of CSF-1, i.e. enhanced survival, was not reversed by PD98059, the specific inhibitor of MEK (mitogen-activated protein kinase/ERK kinase) activation, suggesting that a MEK/ERK pathway is not involved or that the inhibitor cannot effectively suppress sufficient MEK activity under high CSF-1 conditions [35]. We now demonstrate that PD98059 (50 μM) had a partial effect on ox.LDL-induced BMM survival over a 24 h period (Table 2); the lack of a significant effect on the survival response due to optimal CSF-1 is also included. We found previously that CSF-1-stimulated BMM DNA synthesis was partially inhibited by PD98059, suggesting a role, albeit partial, for a MEK/ERK-dependent pathway(s) in the control of the DNA synthesis [35]. A comparison is also made in Table 2 between the effects of PD98059 on ox.LDL-stimulated DNA synthesis and on DNA synthesis stimulated by an optimal CSF-1 concentration, respectively. A partial inhibition was found for both stimuli, suggesting some MEK/ERK involvement. For the experiment in Table 2, the inhibitor was added while ox.LDL was present; a similar result occurred if cells were preloaded with ox.LDL prior to inhibitor addition (results not shown).

Table 2 Effect of PD98059 on ox.LDL-induced BMM survival and DNA synthesis

Growth-arrested CBA-strain BMM (i.e. CSF-1 removed for 24 h) were treated with ox.LDL (50 μg of protein/ml) or with CSF-1 (5000 units/ml), in the absence or presence of PD98059 (50 μM). Viable cell number and DNA synthesis (^{3}H-TdR incorporation) were measured over a 24 h period. Values are means ± S.E.M. from triplicate cultures from a representative experiment, which was repeated a total of six times.

Treatment	Cell number ($\times 10^{-4}$)	^{3}H-TdR incorporation (c.p.m. $\times 10^{-4}$)
$t = 0$	8.8 ± 0.6	–
No addition	1.6 ± 0.2	2.5 ± 0.3
ox.LDL	8.3 ± 0.5	13.5 ± 0.9
ox.LDL + PD98059	6.4 ± 0.5*	5.5 ± 0.3
CSF-1	9.4 ± 0.2	61.9 ± 2.8
CSF-1 + PD98059	8.6 ± 0.4	41.0 ± 2.0

* $P < 0.001$, compared with the ox.LDL-treated group (Student's t test).

Table 3 Effect of PI 3-kinase and p70 S6-kinase inhibitors on ox.LDL-induced BMM survival and DNA synthesis

Growth-arrested CBA-strain BMM (i.e. CSF-1 removed for 24 h) were treated with ox.LDL (50 μg of protein/ml) or with CSF-1 (5000 units/ml), in the absence or presence of wortmannin (100 nM) or rapamycin (30 nM). Wortmannin was also added again at 12 h because of its reported lability. Viable cell number and DNA synthesis (^{3}H-TdR incorporation) were measured over a 24 h period. Values are means $\pm$ S.E.M. from triplicate cultures from a representative experiment which was repeated a total of nine times.

Treatment	Cell number ($\times 10^{-4}$)	^{3}H-TdR incorporation (c.p.m. $\times 10^{-4}$)
$t = 0$	9.7 $\pm$ 0.6	–
No addition	3.4 $\pm$ 0.5	0.58 $\pm$ 0.04
Ox.LDL	9.4 $\pm$ 0.8	8.2 $\pm$ 0.1
Ox.LDL + wortmannin	5.9 $\pm$ 0.5*	2.2 $\pm$ 0.1
Ox.LDL + rapamycin	4.0 $\pm$ 0.2†	0.88 $\pm$ 0.09
CSF-1	9.8 $\pm$ 0.6	48.7 $\pm$ 0.7
CSF-1 + wortmannin	7.2 $\pm$ 0.7	37.1
CSF-1 + rapamycin	7.2 $\pm$ 0.3	39.6 $\pm$ 0.1

* $P < 0.005$ and † $P < 0.001$, compared with the ox.LDL-treated group (Student's t test).

ox.LDL stimulates PI 3-kinase activity

We showed before that CSF-1 stimulated PI 3-kinase activity in BMM in a dose-dependent manner [17,18]. At the time points examined (10 and 30 min), we report that ox.LDL (50 μg of protein/ml) can also enhance its activity in BMM (see the Experimental section), although again to lower levels than those resulting from the action of optimal CSF-1 (5000 units/ml; results not shown). Inhibition by wortmannin or LY294002 has been used widely to delineate cellular roles for PI 3-kinase (see [21] for examples); of particular interest to our studies here is that both drugs have recently been documented as blocking CSF-1-mediated human monocyte survival [21]. We see in Table 3 that wortmannin had a partial effect on ox.LDL-induced BMM survival over a 24 h period; LY294002 (10 μM) gave a similar result (not shown). Wortmannin inhibited slightly the survival promoted by CSF-1 (5000 units/ml; Table 3). We found before that both drugs gave only a small inhibitory effect on the BMM DNA synthesis response to an optimal CSF-1 concentration [49]. However, we see in Table 3 that wortmannin gave a higher inhibition of ox.LDL-induced DNA synthesis; once again its relatively weak effects for optimal CSF-1-mediated stimulation of DNA synthesis [49] can be seen as a comparison. Similar results were obtained if the cells were pulsed with ^{3}H-TdR over a 20–22 h period after ox.LDL (results not shown). LY294002 (10 μM) was also as active as wortmannin on the ox.LDL-induced DNA synthesis response.

Inhibition of a cellular function by rapamycin has been viewed extensively as evidence for a role for p70 S6-kinase activity in that function (see [49] for examples). We found before that CSF-1 stimulated p70 S6-kinase activity in BMM and that rapamycin inhibited only slightly the stimulation of BMM DNA synthesis by an optimal CSF-1 concentration [49]. It can be seen in Table 3, however, that rapamycin, and similarly wortmannin, had stronger inhibitory effects on ox.LDL-mediated BMM survival and DNA synthesis. The weak effect on cell-number maintenance and on the DNA synthesis response in the presence of a high CSF-1 concentration [49] is included for comparison.

One interpretation of the results provided in Table 3 is that a pathway(s) involving PI 3-kinase and p70 S6-kinase is more critical for the induction of BMM cell-cycle progression by ox.LDL than for CSF-1. However, since the degree of CBA-strain BMM DNA synthesis in response to ox.LDL is usually

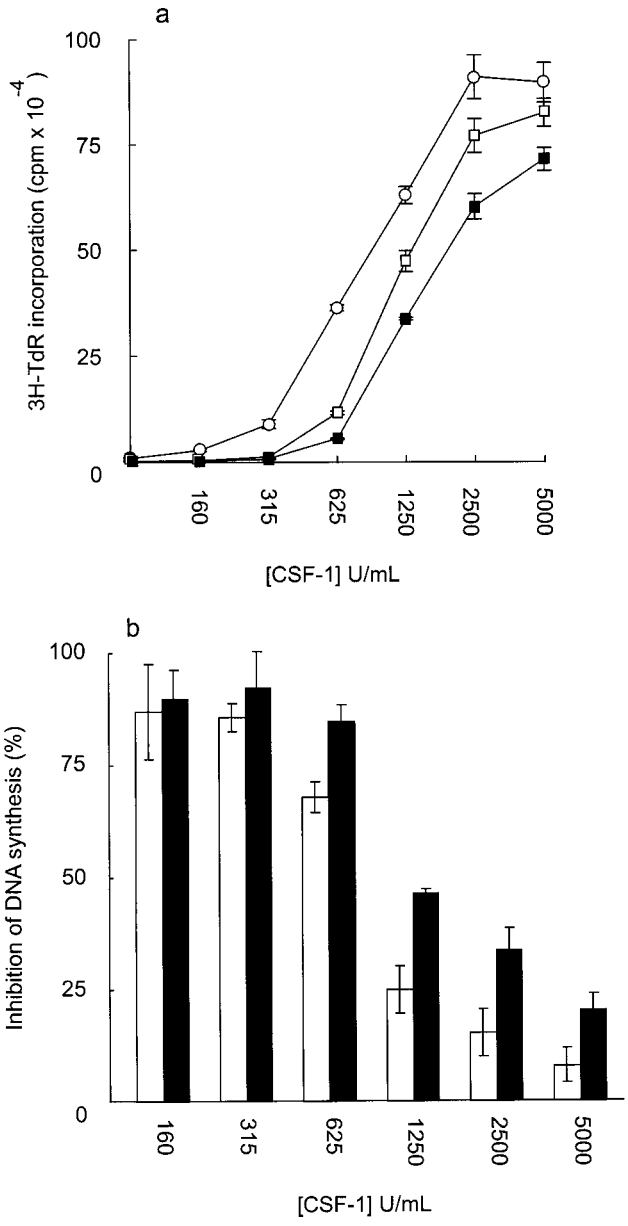

Figure 3 Effect of wortmannin and rapamycin on BMM DNA synthesis at different CSF-1 concentrations

(**a**) Quiescent (growth-arrested) CBA-strain BMM (see the Experimental section) were treated with increasing concentrations of CSF-1 either in the absence (○) or presence of wortmannin (100 nM; □) or rapamycin (30 nM; ■). DNA synthesis was measured after a 24 h ^{3}H-TdR pulse. Values are means $\pm$ S.E.M. from triplicate cultures from a representative experiment that was repeated a total of six times. (**b**) Data in (**a**) were replotted as the percentage inhibition of DNA synthesis by rapamycin (solid bars) or wortmannin (open bars).

less than that occurring in the presence of an optimal CSF-1 concentration ([14]; Tables 1–3), it was reasoned that the potency of wortmannin and rapamycin may be related instead to the strength of the stimulus present in the cultures. We therefore tested the effectiveness of these inhibitors in the presence of different concentrations of CSF-1. For convenience, the data in Figure 3(a) are replotted as the percentage inhibition of DNA synthesis for each drug in Figure 3(b). It can be observed in Figure 3 that wortmannin and rapamycin are indeed more

Table 4 Effect of kinase inhibitors on ox.LDL-induced GM-CSF$^{-/-}$ BMM DNA synthesis

Growth-arrested GM-CSF$^{-/-}$ and GM-CSF$^{+/+}$ BMM (i.e. CSF-1 removed for 24 h) were treated with ox.LDL (50 μg of protein/ml) or with CSF-1 (5000 units/ml), in the absence or presence of wortmannin (100 nM) or PD98059 (50 μM). DNA synthesis was measured over a 24 h period. Values are means $\pm$ S.E.M. from triplicate cultures from a representative experiment, which was repeated a total of four times.

	^{3}H-TdR incorporation (c.p.m. $\times 10^{-4}$)	
Treatment	GM-CSF$^{-/-}$ BMM	GM-CSF$^{+/+}$ BMM
No addition	0.18 $\pm$ 0.06	0.2 $\pm$ 0.02
ox.LDL	20.1 $\pm$ 0.8	13.2 $\pm$ 0.6
ox.LDL + wortmannin	7.8 $\pm$ 0.6	5.5 $\pm$ 0.7
ox.LDL + PD98059	10.7 $\pm$ 0.6	8.4 $\pm$ 0.9

effective in suppressing the CBA-strain BMM DNA synthesis resulting from low CSF-1 concentrations (for example, 625 units/ml) than from high concentrations (for example, 5000 units/ml). In the same experiment, both the DNA synthesis levels and the degree of inhibition of each drug were similar for ox.LDL-treated BMM cultures and for those treated with 315 units/ml CSF-1 (results not shown). Even though wortmannin is relatively labile its effectiveness on high CSF-1 concentrations was not improved if added at time $t = 0$, 4 or 8 h and at either 30 or 100 nM over the 24 h experimental period (results not shown). When wortmannin (100 nM) or rapamycin (30 nM) was added to BMM cultures containing both ox.LDL and a low CSF-1 concentration (160–625 units/ml), leading to a synergistic and dramatic DNA synthesis response [14], then the degree of inhibition of DNA synthesis was again determined by the extent of the DNA synthesis found in the absence of the inhibitors. In other words, the degree of inhibition became more comparable (i.e. weak) with that found at high CSF-1 concentrations (results not shown).

ox.LDL-induced macrophage DNA synthesis is independent of GM-CSF

It is also possible that endogenous GM-CSF could be mediating the above effects of ox.LDL on BMM survival and DNA synthesis; it has in fact been reported that endogenous GM-CSF is required for ox.LDL-induced macrophage DNA synthesis [11,31,33], with a recent claim that a GM-CSF-independent pathway accounts for $< 20\,\%$ of ox.LDL-induced macrophage proliferation [32]. However, using equal numbers of macrophages from GM-CSF gene-deficient (GM-CSF$^{-/-}$) mice (see the Experimental section), we showed that, when the response of GM-CSF$^{-/-}$ BMM to ox.LDL (50 μg of protein/ml) was studied, the increase in DNA synthesis (over 48 h) was no different from that found for GM-CSF$^{+/+}$ BMM ($n = 9$; results not shown). For RPM from GM-CSF$^{-/-}$ mice, DNA synthesis (over 5 days) still occurred, although to a slightly reduced extent compared with that found with RPM from wild-type mice ($n = 7$). The responses of both macrophage populations to optimal CSF-1 (5000 units/ml) were also similar for the two mouse strains. Not surprisingly, given this result for BMM, the ox.LDL-mediated promotion of BMM survival was also similar for GM-CSF$^{-/-}$ and GM-CSF$^{+/+}$ BMM (see also below). In addition, blocking monoclonal antibody to GM-CSF (75 μg/ml; see the Experimental section) did not suppress ox.LDL-induced DNA synthesis for BMM or RPM (results not shown) in spite of being able to suppress the effect of recombinant murine GM-CSF added at an optimal concentration (5 units/ml; see the Experimental section).

The above studies indicate with these approaches that endogenous GM-CSF is not essential for the stimulation of macrophage survival and DNA synthesis by ox.LDL (or by CSF-1), nor does it appear to be making a significant contribution.

Endogenous GM-CSF and wortmannin action

It was recently published that wortmannin suppresses ox.LDL-induced murine macrophage proliferation through inhibition of the action of endogenous GM-CSF and it was therefore concluded that PI 3-kinase is involved downstream in the signalling pathway after GM-CSF induction [32]. However, we have demonstrated here (see above) that in our hands GM-CSF was not necessary for the induction of macrophage survival and DNA synthesis by ox.LDL. We see in Table 4 that wortmannin (100 nM) inhibited the ox.LDL-induced BMM DNA synthesis over a 24 h period to a comparable degree in GM-CSF$^{-/-}$ and GM-CSF$^{+/+}$ BMM, i.e. to levels similar to what we saw for CBA-strain BMM (Table 3). Again, as with CBA-strain BMM (Table 3), we found that wortmannin (100 nM) partially inhibited ox.LDL-promoted BMM survival 24 h after CSF-1 removal to a similar extent in GM-CSF$^{+/+}$ and GM-CSF$^{-/-}$ BMM, i.e. to a similar degree as we demonstrated for CBA BMM (results not shown). For the survival and DNA synthesis responses of both BMM populations, we could find no differences for the effects of LY294002 (10 μM), rapamycin (30 nM) or PD98059 (50 μM). The data for the effect of PD98059 on ox.LDL-induced GM-CSF$^{-/-}$ and GM-CSF$^{+/+}$ BMM DNA synthesis are provided in Table 4.

DISCUSSION

Increased survival and even proliferation of macrophage-derived foam cells [9–14] could provide a significant contribution to their increased numbers occurring in atheromatous plaques [3–8]. Foam cell development, like BMM development *in vitro* and that for certain other macrophage populations *in vivo* [41], has been demonstrated to be CSF-1-dependent [50]. We have suggested before [14] that the increased survival and even proliferation of ox.LDL-'loaded' BMM provides evidence for the above concept regarding enhanced foam cell numbers. Since both ox.LDL and CSF-1 both promote BMM survival and proliferation, with synergy being evident at low CSF-1 concentrations for the DNA synthesis response [14], it is possible that the two stimuli share some common signal-transduction pathway(s).

If the relevant pathways for ox.LDL action are to be elucidated then the roles of endogenous CSF-1 and GM-CSF need to be assessed. As has been reported [11,31–33], it is possible that activation of a particular pathway by ox.LDL may be involved in controlling CSF formation and/or action and may not represent a direct effect of ox.LDL on pathways leading to macrophage growth. Since we have shown previously that ox.LDL and CSF-1 act synergistically in promoting proliferation of BMM [14] and RPM (results not shown), since CSF-1 levels rise in other cell types in response to ox.LDL [24,29], and since macrophages can produce CSF-1 [30], it is not unreasonable to conclude that endogenous CSF-1 may be required for this particular response to ox.LDL. However, the DNA synthesis response to ox.LDL of op/op BMM was similar to that for littermate-control BMM, and blocking antibody to the CSF-1

receptor failed to suppress the DNA synthesis response of CBA-strain BMM and RPM to ox.LDL. All of these findings suggest that endogenous CSF-1 is not necessary for, nor is it making a significant contribution to, ox.LDL-induced macrophage DNA synthesis (and obviously survival). Likewise, we had found before that ox.LDL and GM-CSF synergistically stimulate BMM DNA synthesis [14] and several papers have implicated endogenous GM-CSF as being required and/or as playing a major role in the proliferative response of RPM to ox.LDL [11,31–33]. However, using both GM-CSF gene-deficient mice and blocking antibody to GM-CSF we could find no evidence for a requirement for endogenous GM-CSF in the induction of macrophage DNA synthesis by ox.LDL. Consistent with this lack of an essential requirement for or contribution from either CSF, the steady-state concentration of each CSF in the BMM cultures is presumably less than the lowest concentrations of added CSF providing synergy with ox.LDL for the DNA synthesis response, namely 160 units/ml and 0.1 unit/ml for CSF-1 and GM-CSF, respectively [14]. Supernatants collected 48 h after BMM treatment with 50 μg/ml ox.LDL failed to stimulate BMM DNA synthesis when transferred to untreated BMM (results not shown).

Even though we found that endogenous CSF-1 is not required for the induction of BMM DNA synthesis by ox.LDL, it is still possible that endogenous CSF-1 could be contributing to the activation of a mutual signal-transduction pathway(s). We therefore used op/op BMM to demonstrate that ox.LDL can elevate ERK-1 and ERK-2 activities independently of any endogenous CSF-1. We have also found that, using BMM from op/op GM-CSF$^{-/-}$ mice (i.e. deficient in both CSFs) [14], that GM-CSF was also not required for induction of ERK activity by ox.LDL (results not shown). It was also observed that the stimulation of ERK activity in CBA-strain BMM by ox.LDL was less than that by optimal CSF-1 at the time points examined (Figure 2). PD98059 had a partial effect on ox.LDL-induced BMM survival and DNA synthesis (Table 2), suggesting a partial dependence on a MEK/ERK-dependent pathway(s). At optimal CSF-1 concentrations the inhibitor had no effect on CSF-1-induced survival but a partial effect on the corresponding DNA synthesis response; the inhibitory effect on the latter response was more extensive if the CSF-1 concentration was lowered (results not shown). Whether PD98059 had any effect on CSF-1-induced survival at low CSF-1 concentrations was not examined. The conclusions for the relevance of a MEK/ERK-dependent pathway(s) for CSF-1-mediated BMM survival and proliferation at high CSF-1 concentrations agree with our previous report [35].

As for ERK activity, ox.LDL stimulated a weaker PI 3-kinase response than optimal CSF-1, which was similar to what we found before with low CSF-1 concentrations [17]. Both wortmannin and LY294002 inhibited partially the BMM survival due to ox.LDL (and CSF-1; Table 3, and results not shown). In a prior publication we had found that wortmannin and LY294002, in contrast with many other growth-factor/cell systems, both gave weak inhibition of CSF-1-stimulated BMM DNA synthesis under high (i.e. optimal) CSF-1 concentrations [49]. In contrast, it was found here that these PI 3-kinase inhibitors blocked significantly the stimulation of BMM DNA synthesis by ox.LDL, suggesting that PI 3-kinase may be involved in the DNA synthesis response (Table 3); reduced cell viability would appear to be contributing to this suppression. Our data with GM-CSF$^{-/-}$ BMM do not support the recent conclusion as a general one that wortmannin suppresses ox.LDL-induced murine macrophage proliferation through the action of endogenous GM-CSF and that PI 3-kinase is involved downstream in the signalling pathway after GM-CSF induction [32].

We also found that wortmannin and LY294002 were more effective when the CSF-1 concentrations were lowered, indicating that the inhibitors work best at suppressing BMM DNA synthesis when the cellular stimulus was sub-optimal, namely at low CSF-1 concentration or in the presence of ox.LDL. It was noted that in the presence of wortmannin (and also LY294002 and rapamycin) the BMM spread less and appeared to be less adherent, particularly in the presence of low CSF-1 concentrations or ox.LDL. The relevance of these changes to the effects on the proliferative response (and cell survival) is uncertain. Such a dependence of the degree of inhibition of BMM DNA synthesis on the CSF-1 concentration has been published before with prostaglandin E_2 as the inhibitor [16].

The effects of rapamycin on ox.LDL and CSF-1-induced BMM survival and proliferation are similar to what we found in this study (Table 3) or before [48] with wortmannin, suggesting a connection. Indeed, we reported previously that wortmannin suppressed the CSF-1-stimulated increase in p70 S6-kinase activity [49], indicating that PI 3-kinase activity may lie upstream as in a number of other cellular systems (see [49] for examples). It is likely that the same situation will apply to ox.LDL-treated BMM. It is possible that another pathway(s) becomes more significant as the stimulus increases through elevation of CSF-1 concentrations or when the BMM are treated with ox.LDL and sub-optimal CSF-1; alternatively, this PI 3-kinase/p70 S6-kinase pathway may be activated too strongly for the wortmannin and rapamycin to be as effective.

Both the MTT and XTT [2,3 bis-(2-methoxy-4-nitro-5-sulphophenyl)-2*H*-tetrazolium-5-carboxanilide] formazan methods have been used to monitor the proliferative response of RPM to ox.LDL [10–12,48]. We found a similar rapid induction of MTT formazan in ox.LDL-treated macrophages, presumably reflecting enhanced mitochondrial activity; however, we found no correlation with cell number since this did not increase over the short time period examined, namely 24 h, for BMM and RPM. This dramatic increase in MTT formazan levels was not observed following CSF-1 treatment of BMM and RPM, indicating a difference in the response to the two stimuli. The inhibitory effect of wortmannin (100 nM) on XTT formazan levels over a 4 day period has recently been employed to indicate a role for PI 3-kinase in the induction of RPM growth by ox.LDL [12]. We could not demonstrate such a suppression in the ox.LDL-enhanced MTT formazan levels in RPM over a 24 h period (results not shown); also, in the same experiment (Table 3) wortmannin did not reduce significantly the ox.LDL-enhanced MTT formazan levels in BMM over the 24 h period examined. Our data suggest that this assay cannot be used to monitor macrophage cell number changes in response to ox.LDL.

In conclusion, BMM responses to ox.LDL, for example enhanced survival, DNA synthesis and PI 3-kinase activity, are similar to what is found in response to low CSF-1 concentrations; also the effectiveness of certain signal-transduction inhibitors on the BMM survival and/or DNA synthesis response to both ox.LDL and low CSF-1 concentrations would appear to be similar, with evidence of reduced effectiveness as the strength of the cellular stimulus is raised, for example, by high CSF-1 concentrations.

This work was supported by grants from the National Health and Medical Research Council of Australia, by fellowships (to J.A.H. and W.J.) from the National Health and Medical Research Council of Australia, and by a grant from the National Heart Foundation of Australia (to J.A.H. and W.J.). R. Sallay is thanked for typing the manuscript and N. Wilson for assistance with the ERK assays.

REFERENCES

1 Ross, R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature (London) **362**, 801–809
2 Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. and Witzum, J. L. (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N. Engl. J. Med. **32**, 915–924
3 Villaschi, S. and Spagnoli, L. G. (1983) Autoradiographic and ultrastructural studies on the human fibro-atheromatous plaque. Atherosclerosis **48**, 95–100
4 Gordon, D., Reidy, M. A., Benditt, E. P. and Schwartz, S. M. (1990) Cell proliferation in human coronary arteries. Proc. Natl. Acad. Sci. U.S.A. **87**, 4600–4604
5 Rosenfeld, M. E. and Ross, R. (1990) Macrophage and smooth muscle cell proliferation in atherosclerotic lesions of WHHL and comparably hypercholesterolemic fat-fed rabbits. Arteriosclerosis **10**, 680–687
6 Katsuda, S., Coltrera, M. D., Ross, R. and Gown, A. M. (1993) Human atherosclerosis. IV. Immunocytochemical analysis of cell activation and proliferation in lesions of young adults. Am. J. Pathol. **142**, 1787–1793
7 Rekhter, M. D. and Gordon, D. (1995) Active proliferation of different cell types, including lymphocytes, in human atherosclerotic plaques. Am. J. Pathol. **147**, 668–677
8 Wang, J., Wang, S., Lu, Y., Weng, Y. and Gown, A. M. (1994) GM-CSF and M-CSF expression is associated with macrophage proliferation in progressing and regressing rabbit atheromatous lesions. Exp. Mol. Pathol. **61**, 109–118
9 Yui, S., Sasaki, T., Miyazaki, A., Horiuchi, S. and Yamazaki, M. (1993) Induction of murine macrophage growth by modified LDLs. Arterioscler. Thromb. **13**, 331–337
10 Sakai, M., Miyazaki, A., Hakamata, H., Sasaki, T., Yui, S., Yamazaki, M., Shichiri, M. and Horiuchi, S. (1994) Lysophosphatidylcholine plays an essential role in the mitogenic effect of oxidized low density lipoprotein on murine macrophages. J. Biol. Chem. **269**, 31430–31435
11 Biwa, T., Hakamata, H., Sakai, M., Miyazaki, A., Suzuki, H., Kodama, T., Shichiri, M. and Horiuchi, S. (1998) Induction of murine macrophage growth by oxidized low density lipoprotein is mediated by granulocyte macrophage colony-stimulating factor. J. Biol. Chem. **273**, 28305–28313
12 Martens, J. S., Reiner, N. E., Herrera-Velit, P. and Steinbrecher, U. P. (1998) Phosphatidylinositol 3-kinase is involved in the induction of macrophage growth by oxidized low density lipoprotein. J. Biol. Chem. **273**, 4915–4920
13 Sakai, M., Miyazaki, A., Hakamata, H., Sato, Y., Matsumura, T., Kobori, S., Shichiri, M. and Horiuchi, S. (1996) Lysophosphatidylcholine potentiates the mitogenic activity of modified LDL for human monocyte-derived macrophages. Arterioscler. Thromb. Vasc. Biol. **16**, 600–605
14 Hamilton, J. A., Myers, D., Jessup, W., Cochrane, F., Byrne, R., Whitty, G. and Moss, S. (1999) Oxidized LDL can induce macrophage survival, DNA synthesis, and enhanced proliferative response to CSF-1 and GM-CSF. Arterioscler. Thromb. Vasc. Biol. **19**, 98–105
15 Bartocci, A., Pollard, J. W. and Stanley, E. R. (1986) Regulation of colony-stimulating factor 1 during pregnancy. J. Exp. Med. **164**, 956–961
16 Vairo, G., Argyriou, S., Bordun, A. M., Whitty, G. and Hamilton, J. A. (1990) Inhibition of the signaling pathways for macrophage proliferation by cyclic AMP. Lack of effect on early responses to colony stimulating factor-1. J. Biol. Chem. **265**, 2692–2701
17 Yusoff, P., Hamilton, J. A., Nolan, R. D. and Phillips, W. A. (1994) Haematopoietic colony stimulating factors CSF-1 and GM-CSF increase phosphatidylinositol 3-kinase activity in murine bone marrow-derived macrophages. Growth Factors **10**, 181–192
18 Kanagasundaram, V., Jaworowski, A. and Hamilton, J. A. (1996) Association between phosphatidylinositol-3 kinase, Cbl and other tyrosine phosphorylated proteins in colony-stimulating factor-1-stimulated macrophages. Biochem. J. **320**, 69–77
19 Jaworowski, A., Christy, E., Yusoff, P., Byrne, R. and Hamilton, J. A. (1996) Differences in the kinetics of activation of protein kinases and extracellular signal-related protein kinase 1 in colony-stimulating factor 1-stimulated and lipopolysaccharide-stimulated macrophages. Biochem. J. **320**, 1011–1016
20 L'Allemain, G. (1994) Deciphering the MAP kinase pathway. Progr. Growth Factor Res. **5**, 291–334
21 Kelley, T. W., Graham, M. M., Doseff, A. I., Pomerantz, R. W., Lau, S. M., Ostrowski, M. C., Franke, T. F. and Marsh, C. B. (1999) Macrophage colony-stimulating factor promotes cell survival through Akt/protein kinase B. J. Biol. Chem. **274**, 26393–26398
22 Rosenfeld, M. E., Yla-Herttuala, S., Lipton, B. A., Ord, V. A., Witztum, J. L. and Steinberg, D. (1992) Macrophage colony-stimulating factor mRNA and protein in atherosclerotic lesions of rabbits and humans. Am. J. Pathol **140**, 291–300
23 Salomon, R. N., Underwood, R., Doyle, M. V., Wang, A. and Libby, P. (1992) Increased apolipoprotein E and c-fms gene expression without elevated interleukin 1 or 6 mRNA levels indicates selective activation of macrophage functions in advanced human atheroma. Proc. Natl. Acad. Sci. U.S.A **89**, 2814–2818
24 Rajavashisth, T. B., Andalibi, A., Territo, M. C., Berliner, J. A., Navab, M. and Fogelman, A. M. (1990) Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. Nature (London) **344**, 254–257
25 Sieff, C. A., Neimeyer, C. M., Mentzer, S. J. and Faller, D. V. (1988) Interleukin-1, tumor necrosis factor, and the production of colony-stimulating factors by cultured mesenchymal cells. Blood **72**, 1316–1323
26 Schrader, J. W., Moyer, C., Ziltener, H. J. and Reinisch, C. L. (1991) Release of the cytokines colony-stimulating factor-1, granulocyte-macrophage colony-stimulating factor, and IL-6 by cloned murine vascular smooth muscle cells. J. Immunol. **146**, 3799–3808
27 Filonzi, E. L., Zoellner, H., Stanton, H. and Hamilton, J. A. (1993) Cytokine regulation of granulocyte-macrophage colony stimulating factor and macrophage colony-stimulating factor production in human arterial smooth muscle cells. Atherosclerosis **99**, 241–252
28 de Villiers, W. J., Fraser, I. P., Hughes, D. A., Doyle, A. G. and Gordon, S. (1994) Macrophage-colony-stimulating factor selectively enhances macrophage scavenger receptor expression and function. J. Exp. Med. **180**, 705–709
29 Rajavashisth, T. B., Yamada, H. and Mishra, N. K. (1995) Transcriptional activation of the macrophage-colony stimulating factor gene by minimally modified LDL. Involvement of nuclear factor-kappa B. Arterioscler. Thromb. Vasc. Biol **15**, 1591–1598
30 Becker, S., Warren, M. K. and Haskill, S. (1987) Colony-stimulating factor-induced monocyte survival and differentiation into macrophages in serum-free cultures. J. Immunol. **139**, 3703–3709
31 Matsumura, T., Sakai, M., Matsuda, K., Furukawa, N., Kaneko, K. and Shichiri, M. (1999) Cis-acting DNA elements of mouse granulocyte/macrophage colony-stimulating factor gene responsive to oxidized low density lipoprotein. J. Biol. Chem. **274**, 37665–37672
32 Biwa, T., Sakai, M., Matsumura, T., Kobori, S., Kaneko, K., Miyazaki, A., Hakamata, H., Horiuchi, S. and Shichiri, M. (2000) Sites of action of protein kinase C and phosphatidylinositol 3-kinase are distinct in oxidized low density lipoprotein-induced macrophage proliferation. J. Biol. Chem. **275**, 5810–5816
33 Sakai, M., Biwa, T., Matsumura, T., Takemura, T., Matsuda, H., Anami, Y., Sasahara, T., Kobori, S. and Shichiri, M. (1999) Glucocorticoid inhibits oxidized LDL-induced macrophage growth by suppressing the expression of granulocyte/macrophage colony-stimulating factor. Arterioscler. Thromb. Vasc. Biol. **19**, 1726–1733
34 Vairo, G. and Hamilton, J. A. (1985) CSF-1 stimulates Na^+K^+-ATPase mediated $86Rb^+$ uptake in mouse bone marrow-derived macrophages. Biochem. Biophys. Res. Commun. **132**, 430–437
35 Jaworowski, A., Wilson, N. J., Christy, E., Byrne, R. and Hamilton, J. A. (1999) Roles of the mitogen-activated protein kinase family in macrophage responses to colony stimulating factor-1 addition and withdrawal. J. Biol. Chem. **274**, 15127–15133
36 Hamilton, J. A., Vairo, G. and Lingelbach, S. R. (1988) Activation and proliferation signals in murine macrophages: stimulation of glucose uptake by hemopoietic growth factors and other agents. J. Cell. Physiol. **134**, 405–412
37 Vairo, G. and Hamilton, J. A. (1988) Activation and proliferation signals in murine macrophages: stimulation of Na^+,K^+-ATPase activity by hemopoietic growth factors and other agents. J. Cell. Physiol. **134**, 13–24
38 Tushinski, R. and Stanley, E. R. (1985) The regulation of mononuclear phagocyte entry into S phase by the colony stimulating factor CSF-1. J. Cell. Physiol. **122**, 221–228
39 Stanley, E., Lieschke, G. J., Grail, D., Metcalf, D., Hodgson, G., Gall, J. A., Maher, D. W., Cebon, J., Sinickas, V. and Dunn, A. R. (1994) Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. Proc. Natl. Acad. Sci. U.S.A. **91**, 5592–5596
40 Campbell, I. K., Rich, M. J., Bischof, R. J., Dunn, A. R., Grail, D. and Hamilton, J. A. (1998) Protection from collagen-induced arthritis in granulocyte-macrophage colony-stimulating factor-deficient mice. J. Immunol. **161**, 3639–3644
41 Cecchini, M. G., Dominguez, M. G., Mocci, S., Wetterwald, A., Felix, R., Fleisch, H., Chisholm, O., Hofstetter, W., Pollard, J. W. and Stanley, E. R. (1994) Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. Development **120**, 1357–1372
42 Hamilton, J. A., Chan, J., Byrne, R. J., Bischof, R. J., Jaworowski, A. and Kanagasundaram, V. (1998) MRL/lpr and MRL+/+ macrophage DNA synthesis in the absence and the presence of colony-stimulating factor-1 and granulocyte-macrophage colony-stimulating factor. J. Immunol. **161**, 6802–6811
43 Hamilton, J. A., Vassalli, J. D. and Reich, E. (1976) Macrophage plasminogen activator: induction by asbestos is blocked by anti-inflammatory steroids. J. Exp. Med. **144**, 1689–1694
44 Wilson, N. J., Jaworowski, A., Ward, A. C. and Hamilton, J. A. (1998) cAMP enhances CSF-1-induced ERK activity and c-fos mRNA expression via a MEK-dependent and Ras-independent mechanism in macrophages. Biochem. Biophys. Res. Commun. **244**, 475–480

45 Brown, A. J., Dean, R. T. and Jessup, W. (1996) Free and esterified oxysterol: formation during copper-oxidation of ow density lipoprotein and uptake by macrophages. J. Lipid Res. **37**, 320–335
46 Brown, A. J., Mander, E. L., Gelissen, I. C., Kritharides, L., Dean, R. T. and Jessup, W. (2000) Cholesterol and oxysterol metabolism and subcellular distribution in macrophage foam cells. Accumulation of oxidized esters in lysosomes. J. Lipid Res. **41**, 226–237
47 Sudo, T., Nishikawa, S., Ogawa, M., Kataoka, H., Ohno, N., Izawa, A., Hayashi, S. and Nishikawa, S. (1995) Functional hierarchy of c-kit and c-fms in intramarrow production of CFU-M. Oncogene **11**, 2469–2476
48 Martens, J. S., Lougheed, M., Gomez-Munoz, A. and Steinbrecher, U. P. (1999) A modification of apolipoprotein B accounts for most of the induction of macrophage growth by oxidized low density lipoprotein. J. Biol. Chem. **274**, 10903–10910
49 Hamilton, J. A., Byrne, R., Whitty, G., Vadiveloo, P. K., Marmy, N., Pearson, R. B., Christy, E. and Jaworowski, A. (1998) Effects of wortmannin and rapamycin on CSF-1-mediated responses in macrophages. Int. J. Biochem. Cell Biol. **30**, 271–283
50 Smith, J. D., Trogan, E., Ginsberg, M., Grigauz, C., Tian, J. and Miyata, M. (1995) Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. Proc. Natl. Acad. Sci. U.S.A. **92**, 8264–8268

Received 2 August 2000/13 November 2000; accepted 1 December 2000