Fresh mouse peritoneal macrophages have low scavenger receptor activity

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Abstract Peritoneal macrophages are easily isolated by lavage, suggesting that they are either nonadherent or weakly adherent in situ. Cultured macrophages express class A scavenger receptors (SCR), which mediate Ca^{2+}-independent adhesion in vitro. We examined fresh peritoneal macrophages from mice and from women with endometriosis to determine whether the adherence of these cells was associated with increased expression of class A SCR. Fresh human macrophages were not immunoreactive to SCR antibodies; however, SCR immunoreactivity increased with time in culture. Fresh mouse and human macrophages took up minimal amounts of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI)-acetyl-low density lipoproteins (Ac-LDL), a class A SCR ligand. Murine macrophages in culture for 24–72 h internalized four times more Ac-LDL than fresh cells. Cells cultured for 2 days incorporated 3.2 times more [^{14}C] oleate than freshly isolated cells (55.7 ± 7.9 versus 17.6 ± 3.0 nmol/mg cell protein). In contrast to SCR activity, mouse macrophage SCR mRNA expression was similar in freshly isolated macrophages and those cultured for 3 days. These results suggest that peritoneal macrophages express only low levels of SCR activity in situ and that posttranscriptional regulation after isolation leads to an increase in SCR activity that correlates with adherence of the macrophages in vitro.—**Kim, J. G., C. Keshava, A. A. Murphy, R. E. Pitas, and S. Parthasarathy.** Fresh mouse peritoneal macrophages have low scavenger receptor activity. *J. Lipid Res.* 1997. **38:** 2207–2215.

Supplementary key words acetyl LDL • adhesion • macrophage • modified lipoproteins

Monocytes express only low levels of class A scavenger receptors (SCR). Receptor activity is increased dramatically after adhesion and differentiation of monocytes (1, 2), and after phorbol ester-mediated differentiation of mononuclear cell lines (3). These receptors have a broad ligand specificity, binding and metabolizing modified lipoproteins, such as acetyl (Ac) and oxidized (Ox) low density lipoproteins (LDL), as well as bacterial lipopolysaccharide, a platelet-derived macrophage-binding proteoglycan, certain polynucleotides, and a number of sulfated polysaccharides (4–9). Macrophages in vitro and cells stably transfected to express SCR internalize Ac-LDL and Ox-LDL, and accumulate cytoplasmic cholesteryl ester droplets similar to those in foam cells of atherosclerotic lesions (10–12). The receptor is expressed by foam cells in atherosclerotic lesions (13) and is thought to contribute to their accumulation of lipoprotein-derived cholesterol.

Mouse peritoneal macrophages have been used to study the metabolism of modified lipoproteins (14). While evidence is mounting to indicate the presence of alternate receptors for the uptake of Ox-LDL (15–17), including CD36 (18–22) and FcγRII-B2 (23), the class A SCR accounts for a substantial amount of the metabolism of modified lipoproteins by peritoneal macrophages (11). Mouse peritoneal macrophages have also been used to study the expression (24) and activity of the SCR (14).

Recent studies demonstrate that the class A SCR may also be involved in the divalent cation-independent adhesion of macrophages (25–28). In these studies, the monoclonal antibody 2F8, which is specific for the class A SCR, blocked the Ca^{2+}-independent adhesion of RAW 264 cells, a macrophage cell line, to plastic in the presence of fetal calf serum (FCS) (25). The class A SCR also mediates the calcium-independent binding of human monocyte-derived macrophages to nonenzymatically glycated type IV collagen (28). Further evidence for a role of the class A SCR in cellular adhesion comes from studies of class A SCR-null mice (29), whose peritoneal macrophages are less adhesive in culture and re-

Abbreviations: Ac, acetyl; CE, cholesteryl esters; CHX, cycloheximide; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; LDL, low density lipoprotein; Ox, oxidized; PBS, phosphate-buffered saline.

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main rounded longer than macrophages from control animals (29). Peritoneal macrophages can be isolated by a mild peritoneal lavage, suggesting that they are either nonadherent or adhere weakly in situ. Therefore, they may not express the full complement of adhesion receptors normally expressed by mature macrophages.

To test this hypothesis, we measured the SCR activity of freshly isolated human and mouse peritoneal macrophages. Despite similar levels of class A SCR mRNA, SCR activity was significantly lower in freshly isolated macrophages than in adherent macrophages after 2 days in culture. Adherence of these cells was associated with an increase in SCR activity.

METHODS

Materials

RPMI 1640 medium was purchased from Mediatech. HEPES, β-mercaptoethanol, penicillin, streptomycin, polyinosinic acid, TRI reagent, Sephadex G-50, goat anti-rabbit IgG, goat anti-mouse IgM, fast red, and cycloheximide (CHX) were purchased from Sigma Chemical Co. Polyclonal antiserum (R3B) against a synthetic peptide corresponding to amino acids 103–119 of the bovine SCR was produced in rabbit. HAM-56 antibody was purchased from Dako Corp. [α-^{32}P]-dCTP was purchased from DuPont NEN, and the primer labeling kit was obtained from Amersham Life Science.

LDL preparation

Blood was collected from a healthy donor, and LDL were prepared from the plasma by ultracentrifugation (30). The LDL were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) as described by Pitas et al. (31). Briefly, 1 mg of LDL was added to 2 ml of lipoprotein-deficient serum followed by the addition of 50 μl of DiI (3 mg/ml) in dimethyl sulfoxide. The solution was then gently vortex-mixed and incubated at 37°C for 15 h. The DiI-labeled LDL were isolated by ultracentrifugation, acetylated with acetic anhydride (32), and dialyzed against phosphate-buffered saline (PBS) overnight. After sterilization with 0.2-μm filters, the DiI-Ac-LDL were kept at 4°C and used within 2 weeks.

Human peritoneal cell (macrophage) culture

Peritoneal cells from subjects with endometriosis were obtained during diagnostic laparoscopy. These are

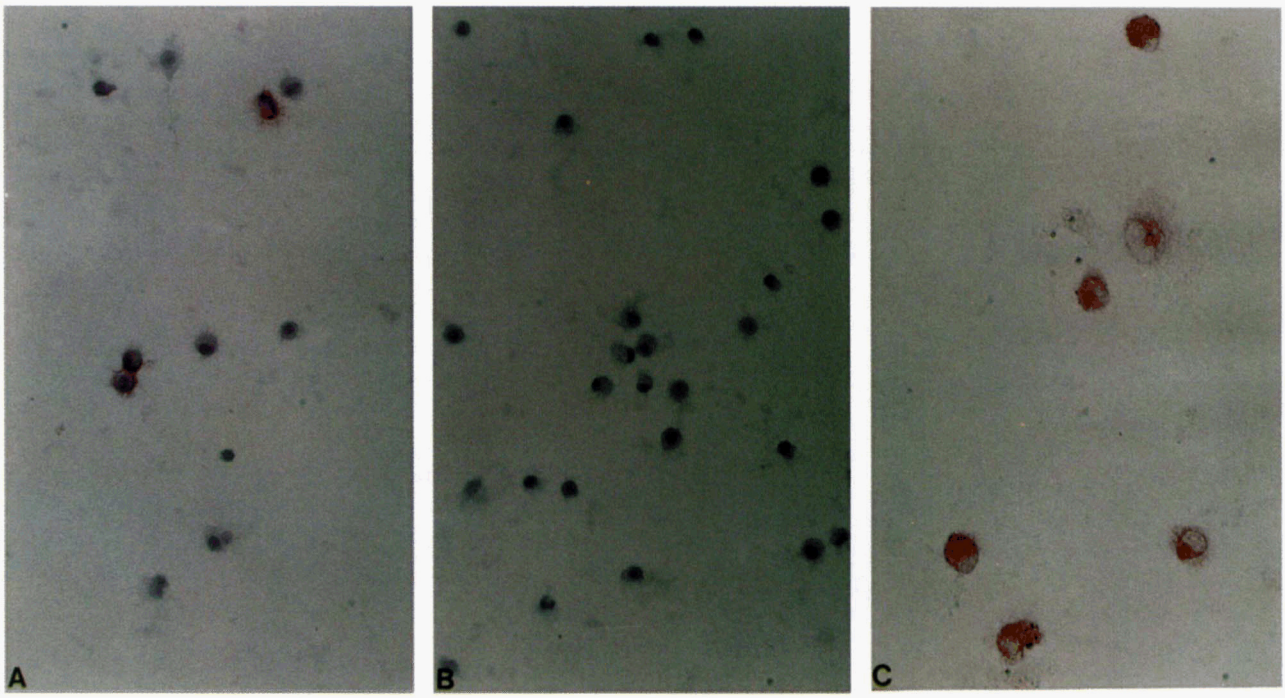

Fig. 1. Immunocytochemical detection of macrophages and SCR protein in human peritoneal cells isolated by peritoneal lavage. Freshly isolated human peritoneal cells were fixed, mounted on poly-L-lysine-treated slides and the presence of macrophages was determined by reactivity to the monoclonal antibody HAM-56 (A). SCR expression was detected with an antipeptide antibody, R3B. Freshly isolated peritoneal macrophages had little SCR protein (B). After 3 days of culture, SCR immunoreactivity increased (C).

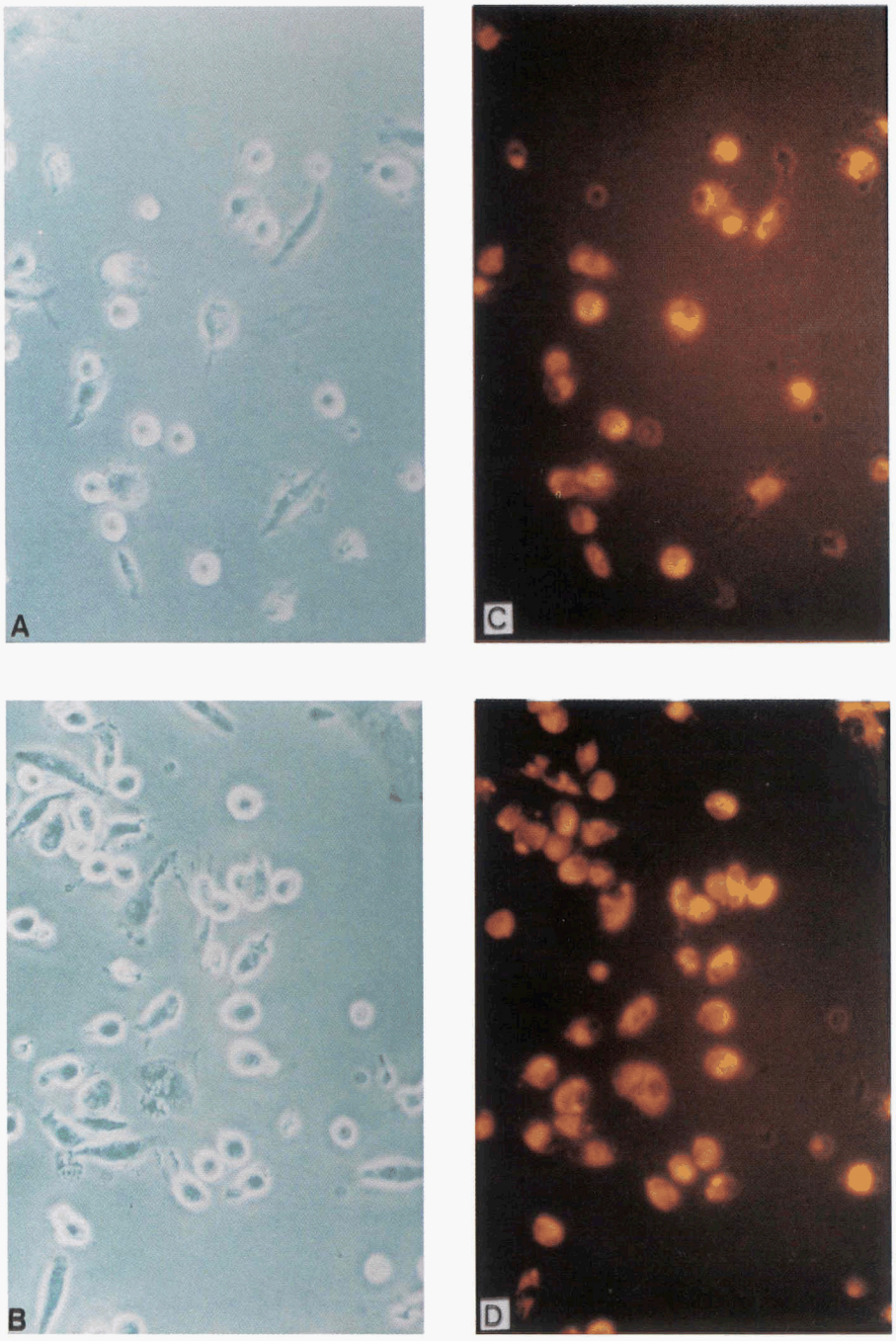

Fig. 2. Peritoneal macrophages cultured for either 2 days (A and C) or 3 days (B and D) were incubated with DiI-Ac-LDL for the last 24 h or 48 h in culture, respectively, and then examined by fluorescence microscopy. Both phase contrast (A and B) and fluorescence (C and D) photomicrographs are shown.

freshly isolated, unadsorbed total cells, which include macrophages at various stages of differentiation, lymphocytes, endometrial cells, and other types of cells. Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 0.1 M HEPES, 50 mM β-mercaptoethanol, penicillin, and streptomycin. For immunocytochemistry, cells were either maintained on multiwell plates (2×10^4/well/0.5 ml) at 37°C in 5% CO_2 or were fixed immediately with formal sucrose (4% paraformaldehyde, 5% sucrose, 20 μmol/L butylated hydroxytoluene, and 1 μmol/L EDTA, pH 7.4). After fixation, freshly isolated nonadherent cells were

mounted on poly-L-lysine-treated slides for immunocytochemical analysis. Adherent cells in culture plates were washed with PBS three times after fixation and analyzed immunocytochemically. After incubation with primary antibodies (either HAM-56 for identification of human macrophages or an anti-SCR antibody R3B) for 2 h at room temperature, the cells were washed and the appropriate secondary antibodies, conjugated with alkaline phosphatase, were added and incubated for 2 h at room temperature. The cells were then washed with Tris-buffered saline, and fast red solution was added; the reaction was terminated after color development. Macrophage SCR activity was assessed qualitatively by using fluorescence microscopy to examine the uptake of DiI-Ac-LDL. In these studies, the cells were cultured for 24 or 48 h in the presence of 5 μg/ml DiI-labeled Ac-LDL with or without a 10-fold excess of either unlabeled Ac-LDL or native LDL.

Mouse peritoneal macrophage culture

Swiss Webster mice weighing 22–25 g were purchased from Harlan. Peritoneal macrophages were recovered by peritoneal lavage with 5 ml of cold RPMI 1640 medium and isolated by centrifugation. The cells were prepared in three different ways. For adherent cells, cells (1×10^6) were plated in triplicate on 6-well tissue culture plates and cultured in RPMI 1640 medium containing 10% FBS, 0.1 M HEPES, 50 mM β-mercaptoethanol, penicillin, and streptomycin for 2 days, as described above. The supernatant was removed, and the cells were washed twice with Hanks' balanced salt solution (HBSS) and used in experiments. Adherent cells to be assayed in a nonadherent environment were washed with HBSS, scraped with a rubber policeman, and transferred in plain RPMI 1640 medium to silicon-treated glass tubes. For nonadherent cells, freshly isolated peritoneal macrophages (1×10^6 cells) were placed directly in siliconized glass tubes containing RPMI 1640 medium. Ac-LDL (12.5 μg/ml in a total volume of 2 ml) and 25 nmol of [^{14}C]oleate (5,000 dpm/nmol), with or without 250 μg of polyinosinic acid, were added to the tubes or plates in triplicate. After 20 min at 37°C in 5% CO_2 for equilibration, the caps were tightened, and the tubes were transferred to the shaker at 37°C for 10 h. Cells in the glass tubes were centrifuged, and the medium was removed. Supernatants from the plastic culture plate were removed. Cells on the plates and in the glass tubes were washed three times with PBS. To determine the amount of label incorporated into cellular cholesteryl esters (CE), lipids were extracted, and thin-layer chromatography was performed. The CE band was scraped from the plate, and [^{14}C]oleate incorporation into CE was quantitated by scintillation counting.

Fluorescence-activated cell sorter (FACS) analysis

The increase in SCR activity after isolation of mouse peritoneal macrophages was further analyzed by FACS analysis. For these studies, peritoneal macrophages were isolated by peritoneal lavage with Ca^{2+}-free PBS, plated in serum-free Dulbecco's modified Eagle's medium (DMEM) for 1.5 h, washed, and incubated in DMEM containing 10% FBS.

DiI-Ac-LDL were added to duplicate plates for a 3-h incubation before the cells were scraped off, fixed, and analyzed by FACS as described (33). Two independent experiments were performed in which cells were incubated with either 5 or 7.5 μg/ml DiI-Ac-LDL. The relative fluorescence intensity of the cells in the absence of DiI was subtracted from all data.

Northern blot analysis

Total RNA was isolated (from fresh macrophages and from macrophages cultured for 3 days) by the TRI reagent method according to the manufacturer's instructions. RNA was quantified by ethidium-bromide staining of 18 and 28S ribosomal RNA. Total RNA (30 μg) was denatured in formamide, fractionated by electrophoresis on 1% agarose gels, and transferred to a nylon membrane. The probe, corresponding to a 795-base *Xba*I and *Hin*dIII fragment of human SCR cDNA, was prepared as described previously (34), randomly labeled with [α-^{32}P]dCTP using a random primer labeling kit (Rediprime, Amersham Corp.). Hybridization and wash procedures were conducted as described previously (35).

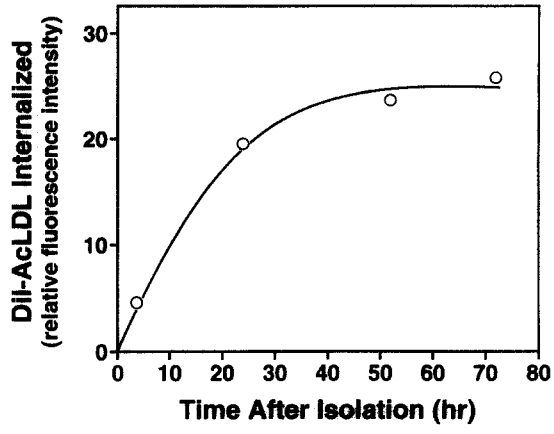

Fig. 3. Effect of time in culture on the internalization of DiI-Ac-LDL by mouse peritoneal macrophages. DiI-Ac-LDL (5 μg/ml) were added to the cells 3 h before harvesting for FACS analysis. The relative fluorescence intensity of the cells in the absence of DiI was subtracted. Each data point is the average of two determinations. This experiment was repeated once with essentially identical results.

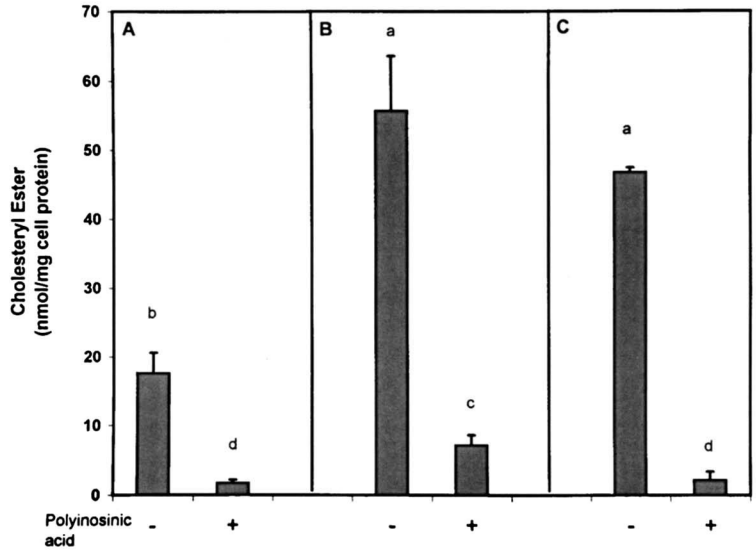

Fig. 4. Effect of adherence of peritoneal macrophages on SCR activity. Freshly isolated mouse peritoneal macrophages were incubated in suspension for the assay of Ac-LDL (12.5 μg/ml in a total volume of 2 ml) to stimulate ACAT activity for 10 h (A). Adherent mouse peritoneal macrophages were maintained for 2 days on tissue culture plates. Cells were scraped and incubated in suspension simultaneously with fresh cells as described in (A) for the assay of Ac-LDL to stimulate ACAT activity for 10 h (B). Adherent mouse peritoneal macrophages maintained for 2 days on tissue culture plates were assayed for the ability of Ac-LDL to stimulate ACAT activity for 10 h on tissue culture plates (C). The incubation was performed in the absence or presence of polyinosinic acid, an SCR competitor. Values are mean ± SD of triplicates. Values with different characters are significantly different ($P < 0.01$).

RESULTS

Fresh peritoneal cells have low levels of SCR protein

Immunocytochemistry was used to examine fresh human peritoneal cells for class A SCR protein expression. Monocyte-macrophages were identified by staining with HAM-56 antibody from freshly isolated human peritoneal cells (**Fig. 1A**), which showed a round morphology similar to that of peripheral blood monocytes. When stained with antibody R3B, which labels SCR, these cells showed practically no immunoreactivity, suggesting the virtual absence of SCR protein (Fig. 1B). This was not due to the inability of the antibody to recognize human SCR because SCR were detected after 3 days of culture (Fig. 1C). Interestingly, round cells tended to be associated with stronger immunostaining.

Uptake of DiI-labeled Ac-LDL by fresh and cultured human macrophages

Human macrophages in culture for 2 or 3 days and incubated during the final 24 or 48 h, respectively, with DiI-Ac-LDL internalized large amounts of fluorescent lipoprotein (**Figs. 2A and B**). Again, macrophages with round morphology, which adhered to the surface but had not yet spread, tended to have intense fluorescence (Figs. 2A and B). These results suggest that adhesion, but not spreading, is associated with the increase in SCR activity. The uptake of DiI-Ac-LDL by SCR was blocked by excess Ac-LDL (10-fold) but not by excess LDL, demonstrating specific internalization of Ac-LDL by the SCR (data not shown). These experiments could not be performed with fresh human macrophages as long incubation with fluorescent DiI-Ac-LDL is needed to visualize the internalized DiI-Ac-LDL. After short (2-h) incubations, nonadherent, freshly isolated cells showed virtually no uptake of DiI-Ac-LDL; however, when cells were cultured for 24 h and then incubated with DiI-Ac-LDL for 2 h, uptake of lipoprotein was apparent (data not shown).

Murine macrophage adherence and SCR activity

To determine whether freshly isolated murine macrophages can actively internalize Ac-LDL, we used FACS

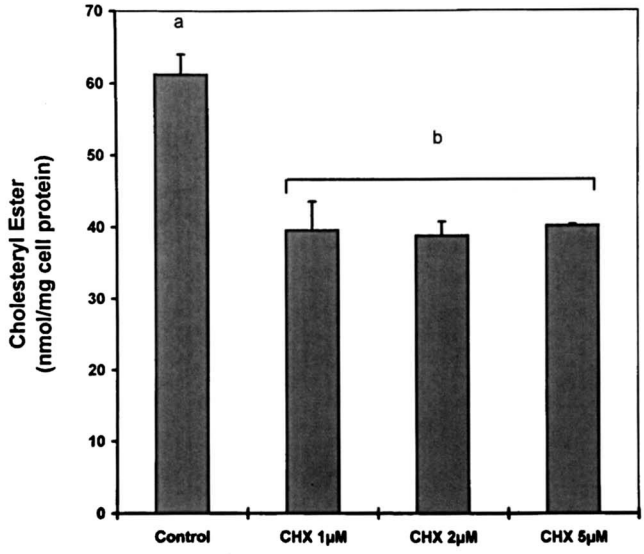

Fig. 5. Effect of the protein synthesis inhibitor CHX on SCR activity in cultured mouse macrophages. Mouse peritoneal macrophages were cultured in the absence of CHX for 24 h and then in the absence (control) or presence of 1, 2, or 5 μM CHX for the next 24 h. Ac-LDL and [^{14}C]oleic acid were added for the second 24 h. Values are mean ± SD of triplicates. Values with different characters are significantly different ($P < 0.01$).

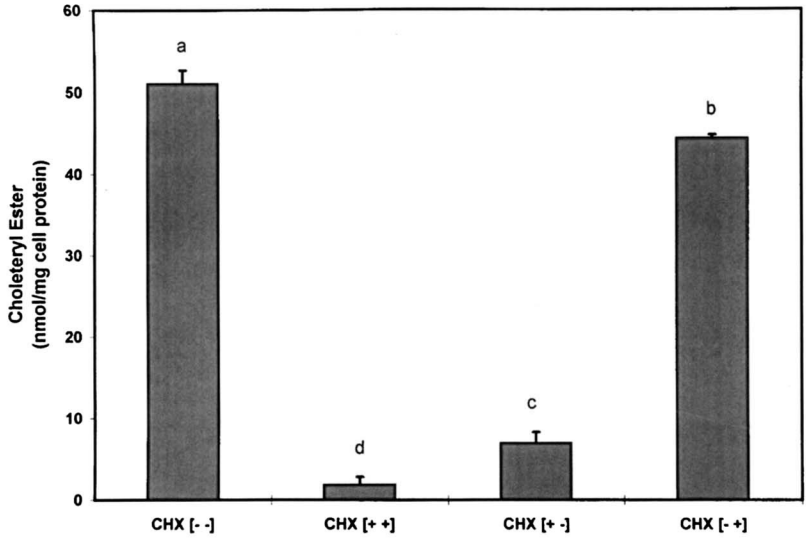

Fig. 6. Effect of the protein synthesis inhibitor CHX on SCR activity in cultured macrophages. Mouse peritoneal macrophages were cultured in the presence or absence of 5 μM CHX for 24 h and then in the absence or presence of 5 μM of CHX for the next 24 h. Ac-LDL and [^{14}C]oleic acid were added for the second 24 h in the presence or absence of CHX. Values are mean ± SD of triplicates. Values with characters a and b are significantly different from values with characters c and d ($P < 0.01$).

analysis to evaluate the internalization of DiI-Ac-LDL by peritoneal macrophages at various times after isolation (**Fig. 3**). The earliest time point evaluated was 4.5 h (1.5 h of adherence followed by a 3-h incubation with DiI-Ac-LDL). Between 4 and 24 h, receptor activity increased ~4-fold and then remained at this level to 72 h (Fig. 3).

To evaluate further the increase in SCR expression in peritoneal macrophages after isolation, we measured the effect of time in culture on the ability of Ac-LDL to stimulate the incorporation of [^{14}C]oleic acid into cellular CE. The freshly isolated cells were maintained in siliconized glass tubes during incubation with Ac-LDL to prevent adherence and differentiation. Cells cultured in tissue culture plates for 48 h were either transferred to siliconized tubes for the assay or assayed on the same plates. CE accumulation was significantly lower in freshly isolated mouse macrophages in siliconized glass tubes (**Fig. 4A**) than in cells cultured on plastic tissue culture plates for 2 days and then transferred to the glass tubes for assay (Fig. 4B) or in cells cultured on plastic wells for 2 days and assayed on the same plate (Fig. 4C) (17.6 ± 3.0 versus 55.7 ± 7.9 and 46.8 ± 0.7 nmol/mg cell protein, respectively, $P < 0.01$). There was no significant difference in CE accumulation in cells assayed on plastic tissue culture plates and those grown on plastic and transferred to the siliconized glass tubes before the assay. Therefore, incubation in siliconized tubes alone had no effect on the uptake of Ac-LDL. The results also validate the use of siliconized glass tubes to determine the lipoprotein uptake under nonadherent conditions. Polyinosinic acid, a competitive inhibitor for the binding of Ac-LDL to the SCR, blocked CE synthesis in both nonadherent and adherent macrophages (Fig. 4).

Taken together, these results demonstrate increased SCR activity as reflected by the uptake of DiI-Ac-LDL and by the ability of Ac-LDL to stimulate cholesterol esterification. SCR activity was low but detectable in the freshly isolated cells, and receptor activity increased with time in culture.

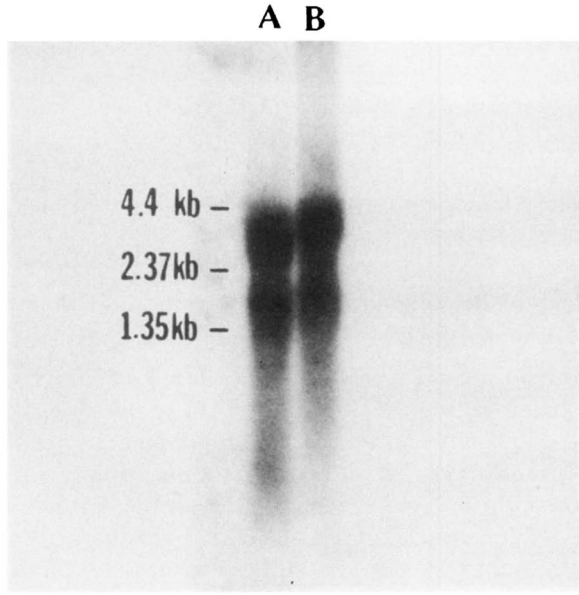

Fig. 7. Northern blot analysis of freshly isolated (lane A) and 3-day cultured (lane B) mouse peritoneal macrophages. Total RNA (30 μg) was fractionated by electrophoresis on 1% agarose gels and transferred to a nylon membrane. The probe, corresponding to a 795-base *Xba*I-*Hin*dIII fragment of human SCR cDNA, was randomly labeled with [α-^{32}P]dCTP. Two bands hybridized to the probe. This experiment was repeated three times with two different probe preparations. There was no difference in the amount of SCR mRNA between freshly isolated and cultured macrophages.

Effect of cycloheximide on SCR activity

The increase in SCR activity over time suggests that SCR protein is actively synthesized during in vitro culture. To determine whether protein synthesis is required for the increase in SCR activity, mouse peritoneal macrophages were cultured in the absence of CHX for 24 h and then in the absence or presence of 1, 2, or 5 μM CHX for the next 24 h to block protein synthesis. As expected, cells cultured in the absence of CHX for 48 h showed high incorporation of [^{14}C]oleate into CE (**Fig. 5**). In cells cultured for 24 h without CHX and then for 24 h in the presence of CHX, CE accumulation was reduced by 30% (Fig. 5). Cellular protein was low when cells were treated with CHX (33.6, 10.3, 7.2, and 6.9 mg/well with 0, 1, 2, and 5 μM of CHX, respectively). The decreased CE accumulation in the presence of CHX could therefore be due to an effect on SCR protein synthesis, to an effect on acyl-CoA:cholesterol acyltransferase (ACAT), or to a decrease in any number of proteins that are involved in cholesterol metabolism.

To clarify the requirement for new SCR protein synthesis in fresh cells, macrophages were cultured in the presence or absence of CHX for the first 24 h and washed with HBSS twice. For the next 24 h, cells were cultured with or without 5 μM CHX in the presence of Ac-LDL and [^{14}C]oleate. Control cells cultured in the absence of CHX showed very good incorporation of [^{14}C]oleate into CE (50 nmol/mg cell protein) (**Fig. 6**). CE accumulation was not significantly reduced in cells incubated with CHX for the second 24 h only, demonstrating that SCR protein is normally synthesized during the first 24 h in culture. CE accumulation was significantly lower in cells cultured with CHX for the initial 24 or 48 h than in cells without CHX from the beginning of culture ($P < 0.01$). As expected, cellular protein synthesis was markedly reduced in cells treated with CHX. The decrease in SCR was not due to a direct effect of CHX on SCR activity, as results from the CHX [− +] group were not significantly different from those in the controls. These results demonstrate that the effect of CHX could not be due to a direct effect on ACAT level, as the CHX [− +] group had no significant difference in oleate incorporation into CE as compared to the CHX [− −] group (Fig. 6). This has also been shown by Tabas, Rosoff, and Boykow (36). Furthermore, these results show that the effect of CHX is not due to cell death, as cells incubated first in the absence and then the presence of CHX [− +] incorporated oleate into CE just as well as the cells not incubated with CHX [− −].

Northern blot analysis

Peripheral blood monocytes and the THP-1 line of monocytes do not express SCR mRNA or activity before differentiation by adherence or treatment with phorbol myristate acetate, respectively. To determine whether the deficiency in SCR activity in fresh peritoneal cells was due to a lack of SCR mRNA, we performed Northern blot analysis. There was no noticeable difference in the expression of SCR mRNA between the freshly isolated cells and adherent cells after 3 days in culture (**Fig. 7**).

DISCUSSION

Murine peritoneal macrophages isolated by peritoneal lavage are used extensively for studies of SCR activity (14). A detailed analysis of the level of expression by freshly isolated cells and the effect of maintenance in culture has not been reported. In the current study, we examined the SCR expression in peritoneal macrophages from mice and from women with endometriosis. Freshly isolated human and murine peritoneal macrophages expressed only low levels of SCR protein, as determined by immunocytochemistry, and low SCR activity, as assessed by both the uptake of DiI-labeled Ac-LDL and the ability of Ac-LDL to stimulate cholesterol esterification. Immunoreactivity and SCR activity (~4-fold) increased with time in culture. Interestingly, the freshly isolated macrophages contained the same amount of SCR mRNA as cells maintained in culture for 3 days. The increase in SCR activity required de novo protein synthesis, because the increase in the ability of Ac-LDL to stimulate cholesterol esterification was blocked by inhibiting protein synthesis with CHX. These studies are the first to suggest that SCR expression by peritoneal macrophages is regulated by post-transcriptional processes and to demonstrate that freshly isolated peritoneal macrophages express only low levels of SCR.

In vitro, the SCR mediates the calcium-independent adhesion of macrophages to tissue culture plastic in the presence of FCS (25), as well as the divalent cation-independent binding to glycated type 4 collagen (28). This binding was specifically blocked by the monoclonal antibody 2F8, which binds the SCR, and by a synthetic peptide corresponding to a portion of the collagen-like domain of the SCR (28). Interestingly, macrophages plated on glycated collagen type 4 had a reduced ability to internalize Ac-LDL, suggesting that the receptors were occupied in adhesion (28). Similarly, in the current study, macrophages that were adherent, but still rounded, appeared to express a higher level of SCR activity than cells that had spread, as assessed both by their ability to internalize DiI-Ac-LDL and by their reactivity to an SCR-specific antibody. These data, therefore, sug-

gest that peritoneal macrophage receptors may participate in adhesion and that receptors involved in adhesion may not be available for ligand binding. The delayed adherence and spreading of macrophages from SCR-null mice provides further evidence for a role of the SCR in adhesion of peritoneal macrophages (29). Data concerning the role of the SCR in mediating adhesion in vivo are more limited (27). The low level of SCR receptor expression by freshly isolated peritoneal macrophages might contribute to their failure to adhere in vivo.

The reason for the low level of SCR expression by peritoneal macrophages in vivo is unknown. It may be that these nonadherent cells represent macrophages recently recruited to the peritoneum that have not fully differentiated. Alternatively, the normal environment of peritoneal macrophages may not be conducive to the terminal differentiation of the cells, and they may be maintained in this state until required for other purposes. Our data do not define the reason for the low level of SCR expression in freshly isolated peritoneal macrophages or the mechanism by which SCR receptor expression is increased when the cells are maintained in culture. As the freshly isolated cells have the same level of SCR mRNA as cells maintained in culture, the low initial levels of receptor protein and the increase in receptor activity with time in culture are likely to result from posttranscriptional regulation. This could result from either regulation of the rate of translation or to changes in the half-life of the SCR protein. The experiments demonstrating the inhibition of SCR up-regulation by CHX do not differentiate these two mechanisms. Regulation of SCR expression by modification of the rate of translation has not been reported, although translational control of expression of numerous other proteins has been demonstrated (for review see 37). Transcriptional and posttranscriptional regulation of SCR by tumor necrosis factor α has been reported; however, the SCR mRNA level was reduced both by a decrease in transcription and by destabilization of the mRNA, leading to more rapid degradation (38). Additional experimentation will be required to determine the mechanism leading to posttranscriptional regulation of SCR activity in peritoneal macrophages.

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