In Vitro–Differentiated Embryonic Stem Cell Macrophages
A Model System for Studying Atherosclerosis-Associated Macrophage Functions

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Abstract—Monocytes/macrophages (Mø) appear to play a critical role in the initiation and progression of atherosclerotic lesions. In this study, we characterized in vitro–differentiated embryonic stem (ES) cell macrophages as a model system for studying atherosclerosis-associated Mø functions. Using immunofluorescence staining and Western analysis, we demonstrate that ES Mø express typical macrophage cell surface markers, as well as the known receptors for modified forms of low density lipoprotein (LDL), including the Mø scavenger receptors (SR-A type I and type II), CD36, and CD68. Differentiated ES Mø specifically bind and degrade \(^{125}\)I-labeled acetylated LDL with high affinity, and their incubation with acetylated LDL (15 \(\mu\)g/mL) for 48 hours produces characteristic “foamy” Mø, as visualized by oil red O staining. ES Mø also express matrix-degrading metalloproteinases (MMP-3, MMP-9), which have been implicated in collagen breakdown in the fibrous cap of atherosclerotic plaques, and secrete cytokines (tumor necrosis factor-\(\alpha\), interleukin-6) in response to inflammatory stimuli. Transfection experiments, using a green fluorescent protein reporter gene, driven by the myeloid-specific promoter, CD11b, demonstrated that ES Mø can also be used to study macrophage-restricted gene expression in vitro. Taken together, these data demonstrate that ES Mø exhibit many properties typical of arterial lesion macrophages. Its ease of genetic manipulation makes it an attractive system for investigations of macrophage functions in vitro. (Arterioscler Thromb Vasc Biol. 1998;18:1647-1654.)

Key Words: atherosclerosis ■ macrophage ■ scavenger receptor ■ foam cell

The current understanding of the pathogenesis of atherosclerosis includes the assignment of a critical role to cells of the monocyte/macrophage lineage. The adherence of Mø to endothelial cells is one of the first events detected in experimental animals fed an atherogenic diet.\(^1\) The subsequent penetration of these cells into the intima of the artery wall and their conversion to cholesterol-loaded foam cells mark one of the earliest events detectable in human atherosclerotic lesions.\(^2\) After macrophage foam cell formation, Mø are thought to contribute to lesion progression through a variety of interactions with other cells of the artery wall that depend on the elucidation of a host of cytokines and growth factors by cells residing in the intima.\(^3\) The number and complexity of these interactions make it difficult to determine which cellular functions are contributing to the progression of atherosclerosis and which might be exploited to interrupt that progression. Homologous recombination, resulting in the selective inactivation of a single gene, has provided a powerful research tool that has proven invaluable in studying many complex disease pathways, including atherosclerosis.\(^4\) Its use can be limited, however, by a requirement for the eliminated gene product in normal animal development, either in a cell type relevant to the disease under investigation or in an entirely unrelated tissue. It therefore would be of value to have a cellular system that permits the results of gene inactivation to be studied in vitro, bypassing the requirement for normal growth and development of an entire animal.

Recent studies have demonstrated that pluripotential mouse embryonic stem (ES) cells can have a gene selectively silenced, followed by differentiation down specific hematopoietic lineages in vitro.\(^5\) This approach provides a genetically tractable cellular system for studying myeloid cell function without requiring the generation of a mouse. In vitro assessment of myeloid cell function then can be performed more easily, because the time and expense required to generate and breed mice is circumvented. In this report, we characterize several macrophage-associated proteins and functions considered relevant to atherosclerosis to determine if they are expressed by ES cells that have been differentiated down the Mø pathway. The studies focus on 2 aspects of Mø function, foam cell formation and secretion of cytokines and metalloproteinases (MMP), that are currently viewed as playing crucial roles in the macrophage’s contributions to atherogenesis.\(^6\) We demonstrate that ES Mø exhibit properties typical of arterial lesion Mø, including the expression of modified LDL receptors (scavenger receptor [SR]-A, CD36, and CD68), capacity for foam cell formation, secretion of MMPs, and expression of inflammatory cytokines. Because ES cells can be easily transfected, and their genetic complement readily altered by homologous recombination, they should prove

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useful in dissecting some of the complex pathways by which macrophages contribute to atherosclerosis.

**Methods**

**Antibodies and Cytokines**

Cell culture supernatants from the following hybridoma cell lines (ATCC) were used as a source of primary mAbs (IgG2b): 2F8, anti-SR-A; F4/80, anti-160-kDa Mø surface antigen; M1/70, anti-CD11b; and 2G2, anti-FcγRII. The anti-CD68 mAb FA/11 was provided by Dr S. Gordon (University of California, San Diego, Calif). The following were used as secondary antibodies: goat anti-rat IgG (fluorescein isothiocyanate [FITC]-conjugated and peroxidase-conjugated) and rabbit anti-guinea pig IgG (peroxidase conjugated) from Sigma Chemical Co. Conditioned media (CM) used as a source of cytokines were: L929 cell CM for CSF-1, X-63 myeloma cell CM for interleukin-3 (IL-3), and 8/24 720 leukemia inhibitory factor (LIF)-D CM for LIF. L929 and X-63 cell lines were obtained from ATCC and the 8/24 720 LIF-D cell line was obtained from Genetics Institute.

**Cell Culture**

ES cells are very sensitive to impurities in culture, which can affect differentiation. To optimize culture conditions, all media, water, and buffers were purchased from Gibco/BRL Life Technologies, and defined FBS was from Hyclone Laboratories Inc. To eliminate contamination from detergents and LPS, all bottles and instruments used were individually packaged, sterile plastic for tissue culture use (Corning Inc). ES cells (J1) were cultured on irradiated embryonal fibroblast feeder layers in DMEM complete (15% FBS, 100 U penicillin/streptomycin) supplemented with 10% FBS supplemented with 15% L929-CM (CSF-1) and 3% IL-3 as previously described.5 The mouse monocytic cell line RAW 264.7 (ATCC) was cultured in Ham’s F12 media (10% FBS; 100 U penicillin/streptomycin), and P388D1 (ATCC) cell line was cultured in DMEM complete media.

**ES Cell Transfection**

Before electroporation, ES cells were trypsinized and washed twice in PBS. A derivative of the plasmid Puc18, containing the CD11b protein (GFP) reporter gene, was linearized by digestion with Mlu I. Before electroporation, ES cells were trypsinized and washed twice in PBS, and then incubated in fresh buffer containing primary Ab for 1 hour (SR-A 2F8 Ab, 10 µg/mL; guinea pig anti-CD36 antisera, a 1:1000; CD68 [FA/11] 1:100). Membranes were washed in TBS-T and incubated with peroxidase-conjugated anti-rat (1:10 000) or anti–guinea pig IgG (1:5000) in TBS-T/5% dry milk. Immunoreactive proteins were visualized using the enhanced chemiluminescence detection system (Dupont NEN, Boston, Mass) and exposed to X-ray film.

**Quantification of AcLDL Uptake and Degradation**

LDL was isolated from fresh human plasma by preparative ultracentrifugation, and acetylated LDL (AcLDL) was prepared by reaction with acetic anhydride and dialysis against phosphate-buffered saline as previously described.10 For foam cell formation, ES-derived Mø (3 × 10⁶) were transferred to serum-free DMEM for 24 hours and incubated with acetic anhydride and serial dialysis against phosphate-buffered saline containing 5% 2-mercaptoethanol and electrophoresed on 8% to 10% linear gradient SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) in the presence of Towbin transfer buffer (25 mMol/L Tris; 192 mMol/L glycine; 20% methanol; pH 8.3) using a wet electrophoretic transfer apparatus. Membranes were blocked in TBS-T (Tris-buffered saline/0.1% Tween-20) containing 5% (wt/vol) nonfat dry milk for 1 hour and then incubated in fresh buffer containing primary Ab for 1 hour (SR-A 2F8 Ab, 10 µg/mL; guinea pig anti-CD36 antisera, a 1:1000; CD68 [FA/11] 1:100). Membranes were washed in TBS-T and incubated with peroxidase-conjugated anti-rat (1:15 000) or anti–guinea pig IgG (1:5000) in TBS-T/5% dry milk. Immunoactive proteins were visualized using the enhanced chemiluminescence detection system (Dupont NEN, Boston, Mass) and exposed to X-ray film.
scribed. CM (10 μL) was mixed with an equal volume of nonreducing Laemmli sample buffer and analyzed by nonreducing SDS-PAGE in 7.5% gels containing 0.1% (wt/v) gelatin (Sigma Chemical Co) for MMP-9 (gelatinase), or in 11% gels containing 2 mg/mL casein for MMP-3 (stromelysin). After electrophoresis, the gels were cleared of SDS by incubating for 1 hour with 2 changes of 2.5% (v/v) Triton X-100 (Sigma Chemical Co). Gels were incubated overnight in substrate buffer (50 mmol/L Tris pH 8; 50 mmol/L NaCl; 10 mmol/L CaCl₂; and 0.05% Brij 35) at 37°C and stained with Coomassie Brilliant Blue to reveal protease activity (clear bands).

Measurement of Cytokine Production
ES Mø (5×10⁵) were stimulated with 100 ng/mL Salmonella minnesota (Re 595) lipopolysaccharide (Sigma Chemical Co) for 18 hours, and cell culture supernatants were collected. Tumor necrosis factor-α (TNF-α) and IL-6 in cell supernatants were assessed by ELISA assay (Endogen). Supernatants were diluted 1:10 before analysis, and all samples were measured in triplicate.

Results

ES-Derived Mø Express Mø Surface Markers
Surface marker staining and phagocytic studies confirmed that embryoid bodies cultured with myeloid growth factors strongly favored Mø differentiation. A single embryoid body produced approximately 4×10⁵ cells that were confirmed by cell surface expression of CD11b to be of the myeloid lineage (Figure 1). More specifically, >90% of cells isolated from secondary embryoid cultures expressed the Mø markers F4/80 and FcγRII as detected by immunofluorescence (Figure 1). Specificity of staining was confirmed by the absence of staining in cultures in which the primary Ab was omitted or replaced with an isotype matched IgG (data not shown). To assess the phagocytic capacity of these Mø, the ability to phagocytose latex beads was tested. After 2 hours’ incuba-
tion, latex beads were abundant in the Mø cytoplasm confirming the phagocytic function of the ES Mø (Figure 2).

ES-Derived Mø Express the Modified LDL Receptors SR-A, CD36, and CD68

To evaluate the suitability of ES-differentiated Mø for the study of foam cell formation, we characterized the complement of modified LDL receptors expressed by ES Mø. Four specific receptors for modified forms of LDL, all members of the broad family of scavenger receptors, have been implicated in this process. These receptors, the macrophage SR-A type I and type II, CD36, and macrosialin (CD68), share the common property of binding a broad array of ligands with high affinity. Unlike the LDL receptor, however, macrophage SRs are not downregulated by a rise in intracellular cholesterol content. Their activity therefore can result in excessive accumulation of cholesteryl ester in the cytoplasm, giving the Mø a characteristic foamy appearance. Western blot analysis demonstrated that ES-differentiated Mø express the 4 known modified LDL receptors (Figure 3).

The 2F8 mAb detected a single protein of approximately 77 kDa corresponding to the SR-A monomer in ES Mø, ES Mø foam cells and the RAW Mø cell line (Figure 3). Furthermore, cell surface expression of the SR-A class receptors was demonstrated by indirect immunofluorescent staining using the 2F8 mAb and was detected in approximately 80% of ES-derived Mø (Figure 1). This result is in agreement with previous in vivo observations that demonstrated heterogeneity of SR-A expression in tissue Mø populations. Because the 2F8 mAb recognizes sequences common to SR-A type I and type II, we confirmed that ES Mø express both SR-A isoforms by reverse transcription (RT)-PCR analysis using type I–specific and type II–specific primer sets (data not shown).

Protein expression of CD36 by ES Mø was confirmed by Western blot analysis. The polyclonal anti-CD36 guinea pig antiserum detected a single protein of approximately 88 kDa in ES Mø and ES Mø foam cells, which comigrated with a similarly sized protein in the RAW Mø cell line (Figure 3). Expression of CD68 by ES Mø and ES Mø foam cells was demonstrated using the FA/11 mAb, which recognized a single protein of approximately 100 kDa. The RAW Mø cell line produced a protein of apparent equal molecular mass (Figure 3). Together, these results demonstrate that ES-derived Mø and ES Mø foam cells express the 4 well-characterized receptors for modified LDL, making them a suitable cellular system for the study of foam cell formation.

ES-Derived Mø Bind and Degrade Modified LDL

Degradation of modified LDL via scavenger receptors results in increased cellular cholesterol content and the formation of cholesteryl ester droplets in the cytoplasm. Incubation of ES Mø in the presence of 15 μg/mL AcLDL for 48 hours resulted in the formation of characteristic foam cells in vitro. Oil red O staining demonstrated that >95% of ES Mø incubated with AcLDL exhibited exaggerated cytoplasmic lipid accumulation, causing a foamy appearance (Figure 4A). As expected, ES Mø incubated with native LDL did not accumulate lipid (Figure 4B). To evaluate the capacity of differentiated ES Mø to degrade modified LDL, we incubated ES Mø or the monocytic cell line P388D1 with 10 μg/ml 125I-AcLDL in the presence or absence of SR ligand competitors. Detected after a standard 5-hour incubation, degradation of 125I-AcLDL by ES Mø exceeded that of P388D1, a Mø cell line commonly used in studies of SR-A degradation (Figure 4C). As expected, degradation of 125I-AcLDL was inhibited by the addition of cold AcLDL or the scavenger receptor ligand polyinosinic acid (poly I), but not by native LDL, which does not bind to the scavenger receptor. These data demonstrate that modified LDL uptake by ES Mø is both avid and specific. The disposition of the ingested lipid is similar to that seen in both native macrophages and commonly used, transformed, monocytic cell culture lines.
ES-Mø Express

Matrix-Degrading Metalloproteinases
Plaque Mø overexpress a variety of matrix-degrading metalloproteinases (collagenase, stromelysin, and gelatinase B) that can catabolize the constituents of the arterial extracellular matrix. Because plaque rupture provides the locus at which coronary thrombosis can occur, these observations suggest that the accumulation of macrophages and lipid critically influence the acute clinical manifestations of coronary heart disease. The expression of MMP by ES Mø was therefore examined. RT-PCR analysis revealed that ES Mø constitutively express MMP-3 and MMP-9 but express very little MMP-1 (Figure 5A). By comparison, the RAW Mø cell line expresses MMP-9 but not detectable levels of MMP-1 or MMP-3. Control PCR reactions in the absence of reverse transcriptase yielded no product, eliminating the possibility of genomic contamination. To determine whether ES Mø secrete activated MMPs, we used zymography to analyze CM from these cells. Gelatin zymographic analysis revealed constitutive secretion of a 92-kDa protease corresponding to MMP-9 in ES Mø and ES Mø foam cells (Figure 5B). This activity comigrated with a protease secreted by human peripheral blood monocyte–derived Mø that constitutively express MMP-9. We did not detect any MMP-3 activity in conditioned media from ES Mø or ES Mø foam cells by casein zymography (data not shown). It is likely that the MMP-3 activity in our samples is below the limits of detection of this assay due to the relative insensitivity of casein zymography.

ES-Mø Elaborate Cytokines in Response to Inflammatory Stimuli
Mø are a primary source of inflammatory cytokines within atherosclerotic lesions. Through modulation of such functions as leukocyte recruitment, autocrine/paracrine induction of cytokines, and synthesis/degradation of vascular extracellular matrix constituents, cytokines can influence both lesion initiation and progression. Therefore, we tested the capacity of ES Mø to produce cytokines in response to an inflammatory stimulus. ES Mø, but not undifferentiated ES cells, secreted abundant amounts of TNF-α and IL-6 into the cell culture supernatant in response to LPS (Figure 6). As expected, unstimulated ES Mø did not elaborate TNF-α or IL-6.

Myeloid-Specific Promoter CD11b Drives Mø-Specific Expression of the Reporter Gene, GFP, in Differentiated ES Mø
Genetic manipulation of ES cells and subsequent differentiation into Mø provides a system to overcome the poor...
transfectability of Mø. To demonstrate differential gene expression during the development of totipotent ES cells into differentiated Mø, we used the myeloid promoter, CD11b, driving a GFP reporter gene. ES cells stably transfected with the CD11b/GFP plasmid did not express GFP before differentiation (data not shown). Expression of GFP was first detectable by fluorescence microscopy in differentiating embryoid bodies (10 days) cultured in the presence of macrophage growth factors. Fully differentiated ES Mø were harvested at 20 days and stained for the presence of CD11b and the Mø-specific antigen F4/80. Endogenous cell surface expression of CD11b, as demonstrated by immunostaining, correlated with GFP activity in differentiated ES cells (Figure 7A and 7B). Furthermore, the vast majority of isolated GFP expressing cells also expressed F4/80 Ag, confirming that these cells were Mø (Figure 7C and 7D). Thus, on differentiation of transfected ES cells, cell-specific transgene expression can be stably achieved in the resultant Mø.

Discussion

The current clinical understanding of atherosclerosis recognizes the contribution of the Mø as paramount to the initiation, progression, and eventual rupture of atherosclerotic plaque. Recruitment of the Mø to the arterial wall, and its subsequent engorgement with lipid to form foam cells, is thought to represent the critical initiation step in the development of atherosclerotic plaque. In the more advanced, complex lesion, the Mø foam cell influences the formation of the lipid-rich necrotic core and secretes MMPs. The latter have been implicated in extracellular matrix breakdown in the fibrous cap and thus may contribute to plaque instability. Given this postulated central role of the Mø in the pathogenesis of atherosclerosis, elucidation of the mechanisms by which macrophages participate in this process is likely to be important for the development of improved treatments for coronary artery disease. One of the primary obstacles to achieving this goal has been the availability of a suitable monocyte/Mø cellular system for these studies. Widely used cell lines are often criticized as being imprecise models of normal cells because their transformed state can lead to changes in cellular behavior that do not mimic those of normal, nontransformed cells. Difficulty in genetically manipulating monocytic/Mø cell lines or primary Mø cultures adds to this problem. To find a cellular system that more closely approximates native Mø, yet retains considerable genetic versatility, differentiated pluripotent ES cells were examined. Previous studies had shown that these cells could

Figure 5. ES Mø express matrix degrading metalloproteinases. A, RT-PCR analysis demonstrating constitutive expression of MMP-3 and MMP-9, but little of MMP-1 by ES-differentiated Mø. By comparison, the Mø cell line RAW 264.7 demonstrates constitutive expression of MMP-9, but not of MMP-1 or MMP-3. B, Gelatin zymogram demonstrating secreted gelatinolytic activity by ES Mø and ES Mø foam cells, which co-migrates with 92 kDa protease expression by human monocyte–derived Mø, consistent with the secretion of MMP-9.

Figure 6. ES Mø secrete TNF-α and IL-6 in response to an inflammatory stimulus. After LPS stimulation (100 ng/mL, 18 hours), ES Mø elaborate TNF-α (A) and IL-6 (B) protein as measured by ELISA assay. As expected, unstimulated ES Mø produced no detectable TNF-α or IL-6.
be differentiated into a variety of cell types, including myeloid and hematopoietic cells.\textsuperscript{21–23}

In this study we characterized ES-differentiated M\textsubscript{o} as a model system. We demonstrated that ES M\textsubscript{o} (1) express the modified LDL receptors SR-A type I and type II, CD36, and CD68; (2) internalize and degrade AcLDL, forming foam cells in vitro; (3) constitutively secrete matrix degrading metalloproteinases implicated in plaque rupture; and (4) secrete cytokines in response to an inflammatory stimulus. In addition, M\textsubscript{o}-restricted reporter gene expression was achieved by differentiating ES cells transfected with a GFP reporter gene regulated by the myeloid-specific promoter CD11b. These data demonstrate that ES M\textsubscript{o} exhibit properties typical of arterial lesion macrophages that would make them useful for in vitro studies of macrophage functions that are currently believed to be important in atherogenesis.

There are several important advantages inherent in using ES cell–derived macrophages as a cell culture system for studying M\textsubscript{o} function. Because the cells are not transformed, and the progenitor cells arising from ES cells are capable of reconstituting the entire hematopoietic compartment of a mouse, they represent a cell culture system that appears to retain the physiological regulation on growth and differentiation that is absent from transformed myelomonocytic cell lines. In addition, the ease of transfection of ES cells may overcome some of the obstacles to gene expression studies that are now impeded by the notoriously inefficient transfections of monocytic cell culture lines. Although these attributes are valuable, the greatest strength of this system is the ability to inactivate genes in a single allele in an ES cell line, convert the line to a homozygously deficient state by antibiotic driven gene conversion, and then examine phenotypic alterations in macrophage function in vitro.\textsuperscript{24} In many transformed cell lines, the combination of polyploid DNA complement and poor transfectability would make this approach too technically demanding.

The properties of ES-derived macrophages outlined above have been exploited recently in studies of transcription factor–regulated macrophage differentiation as well as tissue-specific regulation of expression.\textsuperscript{25,26} Previous studies have not, however, characterized macrophages derived from ES cells to clarify their use for atherosclerosis research. Our results suggest the possibility that several genes expressed in atherosclerotic macrophages might be targeted for elimination in ES cells. Using multiple selection strategies to inactivate multiple genes, investigators should be able to characterize the dependence of critical macrophage functions on several genes whose redundant functions would render a single gene knockout uninformative. For example, the systematic elimination of all known scavenger receptor family members from macrophages could provide insights into the importance of foam cell formation on altering macrophage function in vitro. If multitargeted ES cell lines can also retain their ability to reconstitute a mouse when reimplanted into blastocysts, then in vivo studies of such genetically deficient mice could dramatically facilitate the elucidation of the macrophage’s contributions to atherosclerosis. Studies currently are underway in our laboratory to assess the potential use of these cells in such in vitro and in vivo analyses.

In summary, the work reported in this article has demonstrated that macrophages derived from differentiated ES cells express many gene products that are currently believed to play a critical role in macrophage contributions to the atherosclerotic process. Specifically, ES M\textsubscript{o} are actively
phagocytic and express all commonly used immunohistochemical macrophage cell surface markers, as well as the known receptors for modified lipoprotein uptake (SR-A, CD36, and CD68). These cells retain the capacity for foam cell formation and secrete activatable metalloproteinases that have been implicated in plaque instability and rupture. The technology for altering the genotype of ES cells is now very well established, providing the potential for using genetically altered ES–derived macrophages to dissect the complex behavior of macrophages that is responsible for their involvement in the initiation and progressive development of atherosclerotic lesions.

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