Identification and Isolation of Endothelial Cells Based on Their Increased Uptake of Acetylated-Low Density Lipoprotein

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ABSTRACT Acetylated-low density lipoprotein (Ac-LDL) is taken up by macrophages and endothelial cells via the "scavenger cell pathway" of LDL metabolism. In this report, aortic and microvascular endothelial cells internalized and degraded 7–15 times more [125I]–Ac-LDL than did smooth muscle cells or pericytes. Bound [125I]–Ac-LDL was displaced by unlabeled Ac-LDL, but not unmodified LDL. The ability to identify endothelial cells based on their increased metabolism of Ac-LDL was examined using Ac-LDL labeled with the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'–tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL). When cells were incubated with 10 μg/ml DiI-Ac-LDL for 4 h at 37°C and subsequently examined by fluorescence microscopy, capillary and aortic endothelial cells were brilliantly fluorescent whereas the fluorescent intensity of retinal pericytes and smooth muscle cells was only slightly above background levels. DiI-Ac-LDL at the concentration used for labeling cells had no effect on endothelial cell growth rate. When primary cultures of bovine adrenal capillary cells were labeled with 10 μg/ml of DiI-Ac-LDL for 4 h at 37°C, then trypsinized and subjected to fluorescence-activated cell sorting, pure cultures of capillary endothelial cells could be obtained. Utilizing this method, large numbers of early passage microvascular endothelial cells can be obtained in significantly less time than with conventional methods.

A major problem in the study of microvascular endothelial cells is the identification of the desired cell population and the subsequent isolation of pure cultures. Our currently used method of establishing pure cultures of capillary endothelial cells involves many weeks of "weeding out" nonendothelial cells (1). The weeding technique involves the assumption that the morphology of capillary endothelial cells is similar to other endothelial cells and is thus directed at isolating colonies with those characteristics. Several markers for endothelial cells are routinely used for confirmation that established cell lines are of endothelial origin. These include the presence of factor VIII related antigen (2, 3) and angiotensin converting enzyme (4, 5). Microvascular endothelial cells differ from large vessel endothelial cells in their requirement for additional growth factors and modified surfaces for optimal growth (1), and their response to tumor factors (1, 6).

The receptor-mediated uptake of low density lipoprotein (LDL) by cells has been studied in detail (for a review see reference 7). An alternative pathway for the metabolism of chemically modified lipoproteins has also been described (8) and has been termed the "scavenger cell pathway" of LDL metabolism, due to its occurrence in rodent and canine macrophages (9–11) and human monocytes (12). Various chemical methods for modification of LDL have been used to modify the charge of amino groups on LDL including acetylation (8), acetooxacylation (9), and malondialdehyde treatment (12). These modified lipoproteins are taken up by...
cells by a receptor (10), which differs from the receptor for unmodified LDL in terms of specificity, regulation of cholesterol levels (10, 13), and biochemical properties (13). In addition to monocytes and macrophages, bovine aortic endothelial cells have been demonstrated to metabolize acetylated LDL, but not normal LDL, at an accelerated rate compared with other cell types (14).

Flow cytometric instrumentation allows one to analyze and sort complex cell populations (for a review see reference 15). Using flow cytometry, it is possible to sort cell mixtures based on the differential labeling of various cell types in the mixture. Cells may be specifically labeled based on the differential expression of antigens or on metabolic differences. Lipoproteins labeled with the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI) have been utilized to visualize the uptake of lipoproteins by macrophages and arterial foam cells (16, 17). DiI is a highly lipophilic molecule that can be noncovalently incorporated into lipoproteins and thus has no effect on surface charge. Once a lipoprotein that contains DiI is taken up by a cell, the lipoprotein molecule is acted upon by lysosomal enzymes and the DiI accumulates in lysosomal membranes. Thus, cells that metabolize lipoproteins at different rates will accumulate varying amounts of DiI. In this paper we demonstrate that capillary as well as aortic endothelial cells, but not pericytes or smooth muscle cells possess the scavenger pathway for acetylated-low density lipoprotein (Ac-LDL) metabolism. We further demonstrate that DiI-Ac-LDL can be used to metabolically label endothelial cells thus facilitating their identification by fluorescence microscopy and isolation by fluorescence-activated cell sorting.

MATERIALS AND METHODS

Lipoprotein Isolation and Modification:
LDL (d 1.019-1.063 g ml⁻¹) and lipoprotein deficient serum (d 1.21 g ml⁻¹) were obtained from the serum of fasted human donors by sequential ultracentrifugation using standard techniques (18). LDL was acetylated using acetic anhydride as described (10) and then labeled with the fluorescent probe DiI (Molecular Probes, Junction City, OR) as described by the method of Pitas et al. (16). Ac-LDL was iodinated using [125I]-Ac-LDL, but not normal LDL, at an accelerated rate than both smooth muscle cells and pericytes. Confluent BAECs degraded [125I]-Ac-LDL at a higher rate than preconfluent BAECs as expected from previous studies (14). BCECs were examined only at one density. The specificity of lipoprotein degradation was examined by conducting incubations in the presence of a 20-fold excess of unlabeled LDL or Ac-LDL (Fig. 1 b). Ac-LDL was neutralized by washing the cells once in DME containing 10% calf serum. Prior to sorting, the cells were resuspended in serum-free DME. Cells and collection tubes were kept chilled on ice prior to and during the sorting procedure.

DiI-Ac-LDL-labeled endothelial cells were sorted from other cell types using a Becton-Dickinson FACs IV cell sorter (Mountain View, CA). The 514-nm line of an argon laser was used for excitation. The fluorescence emission above 550 nm was collected. Sampling ports were set using stained BAECs as "positive" and "negative" controls. The scatter gates were set to minimize the contribution of cell pairs and the fluorescence gates were set to eliminate the more highly fluorescent macrophages. Cells were collected into tubes containing 10% calf serum. After the sort was complete, the cells were pelleted, washed once, and placed at various dilutions in gelatin-coated 24 well plates in BCEC growth media. All cell sorting was performed in the cell sorting facilities of the Department of Genetics, Children's Hospital (Boston, MA) under the direction of Dr. Marc Lalande and Mr. Robert Hoffman.

RESULTS

The presence of a scavenger pathway of lipoprotein metabolism in capillary and aortic endothelial cells was examined by incubating cultures with [125I]-Ac-LDL. As shown in Fig. 1 a, BAECs and BAECs degraded Ac-LDL at a rate 7-15 times higher than both smooth muscle cells and pericytes. Confluent BAECs degraded [125I]-Ac-LDL at a higher rate than preconfluent BAECs as expected from previous studies (14). BCECs were examined only at one density. The specificity of lipoprotein degradation was examined by conducting incubations in the presence of a 20-fold excess of unlabeled LDL or Ac-LDL (Fig. 1 b). Ac-LDL but not LDL reduced lipoprotein metabolism by >90%.

To visualize the interaction of Ac-LDL with endothelial cells, cultures were incubated with Dil-Ac-LDL and examined by fluorescence microscopy. Pure cultures of both BCECs and BAECs were brightly stained (Fig. 2). The fluorescence was predominantly punctate with a perinuclear distribution. Low background fluorescence was observed in cultures of smooth muscle cells or pericytes. In mixed cultures of endothelial cells and other cells, endothelial cells were easily distinguished (Fig. 3). When primary cultures of BCECs were stained with Di-Ac-LDL and examined, colonies of endothelial cells were

2 Via, D. P., H. A. Dresel, and A. M. Gotto, Jr., submitted for publication.
3 Orbridge, A., and P. D'Amore, manuscript in preparation.
brightly fluorescent, while all other cell types, except macrophages, exhibited varying levels of background uptake. Macrophages, when present could be easily distinguished from endothelial cells by their shape, granular contents, and much greater fluorescence intensity.

The effect of DiI-Ac-LDL uptake on endothelial cell growth is shown in Fig. 4. No inhibition of cell growth was observed over a period of 7 d when BAECs were labeled with DiI-Ac-LDL prior to trypsinization and plating for the growth curve. Thus the fluorescent probe DiI that accumulates in the cells, is not cytotoxic.

After staining with DiI-Ac-LDL, cultures of cells were analyzed and sorted using a FACS IV cell sorter. As expected, a strong signal was obtained with BAECs while BASMCs gave a weaker fluorescence intensity (Fig. 5 a). The data from these analyses were used to set scatter and fluorescence sampling gates for analysis and sorting of the primary culture. The profile of an 8-d old primary culture from a collagenase digest of bovine adrenal cortex labeled with DiI-Ac-LDL is seen in Fig. 5 b. Quantitation of the profile indicates that the DiI-Ac-LDL-labeled endothelial cells represented ~7% of this cell mixture. The endothelial cells were separated from those cells exhibiting lower levels of fluorescence by sorting under sterile conditions and were subsequently grown in culture (BCEC-S1s). Initially these cultures were ~95% endothelial cells based on visual examination. Allowing the cells to grow in culture for 2 wk at this point resulted in higher numbers of cells for subsequent sorts, 1 × 10^4 BCEC-S1s and 5 × 10^5 BCEC-S2s were used. The percent endothelial cells in each preparation at the time of the sort is summarized in Table 1.

The cultures obtained from the second sort (BCEC-S2s) were grown in wells of 24 well plates until confluent and then expanded for growth and marker antigen analysis. The BCEC-S2s had a doubling time of 4.2 d as compared with 3.7 d for BCECs isolated by the "weeding and feeding" protocol. Fig. 6 shows micrographs of these cells. As shown, all of the cells take up high amounts of DiI-Ac-LDL and are positive for the presence of factor VIII R:Ag. DiI-Ac-LDL consistently labeled endothelial cells much more uniformly than did antibodies against factor VIII R:Ag.

DISCUSSION

In this report, we have demonstrated that capillary endothelial cells degrade iodinated Ac-LDL at a much higher rate than other microvascular cells. Capillary endothelial cells metabolize Ac-LDL at the same high rate as shown previously for aortic endothelial cells (14). Microvascular pericytes degrade much lower levels, in common with those shown for aortic smooth muscle cells (14).

On the basis of this differential uptake of iodinated Ac-LDL, we have used fluorescent-labeled Ac-LDL to metabolically label capillary endothelial cells, facilitating both their identification and isolation. When primary cultures derived from bovine adrenocorticoid microvessels were labeled with DiI-Ac-LDL, only the endothelial cells were labeled. We have successfully used this labeling procedure in conjunction with fluorescence activated cell sorting, to isolate pure capillary endothelial cells from mixed primary cultures derived from bovine adrenal cortex.

The fluorescent probe used, DiI, was very well suited for these studies. Due to its lipophilic nature, once the DiI-Ac-LDL enters the cell and is degraded by lysosomes, it accumulates in the lysosomal membranes (16). The emission spectrum of this fluorescent probe does not overlap with cell autofluorescence and is readily visualized with standard filter sets for rhodamine fluorescence. Furthermore, we have demonstrated that DiI-Ac-LDL has no inhibitory effect on the growth rate of cells that internalize it.

Labeling endothelial cells by the method presented here has numerous advantages over other previously reported endothelial cell specific markers such as anti-factor VIII R:Ag (2, 3) and anti-angiotensin converting enzymes (4, 5). The primary advantage is that the method is highly reproducible and all endothelial cells are labeled. Also, the labeling is performed on live cells with no fixation or permeabilization required for optimal labeling. Furthermore, because the probe is incorporated into lysosomal membranes, the label is not removed from the cells during trypsinization.

Isolating endothelial cells by cell sorting has distinct advantages over other isolation techniques. The technique is fast compared with established procedures. After two sorts and an elapsed time of 4–5 wk we obtained pure microvascular endothelial cell populations from primary cultures that initially contained <10% endothelial cells. In contrast, earlier methods (1) required 3–4 mo to obtain pure cultures. When primary cultures contain a lower percentage of endothelial cells, additional sorts at 2-wk intervals should allow pure endothelial cell cultures to be rapidly obtained. Using this technology, no visual biases such as morphology or growth characteristics are involved in the selection process. Another
Figure 2  Labeling of vascular cells with Dil-Ac-LDL. Phase-contrast (a, c, e, g) and fluorescence (b, d, f, h) micrographs of BAECs (a and b), BCECs (c and d), BASMCs (e and f), and BRPs (g and h) that have been incubated with 10 μg mL⁻¹ Dil-Ac-LDL for 4 h at 37°C. The cells were visualized using a standard rhodamine excitation:emission filter set. Note the abundance of punctate perinuclear fluorescence in the BAECs (b) and BCECs (d). Only background fluorescence is observed in the BASMCs (f) and BRPs (h). Bar, 20 μm. × 250.
advantage is that if desired, a heterogeneous population of endothelial cells derived from multiple microvessels can be obtained for further studies. The cells obtained may be cloned if desired by flow cytometry and single cell deposition or by serial dilution.

Auerbach et al. (23) first demonstrated the feasibility of using a cell sorter to separate endothelial cells from other cell types. They utilized a monoclonal antibody against the cell surface marker, angiotensin converting enzyme to differentiate endothelial cells from other cell types. This method had limitations imposed by the facts that angiotensin converting enzyme is not expressed until after cells reach confluence (24) and their monoclonal antibody bound to some nonendothelial cell types with Fc receptors. In contrast, endothelial cells at all densities degrade Ac-LDL at elevated rates compared with other cell types.

DiI-Ac-LDL is potentially useful in other situations where endothelial cell identification is desired. Ac-LDL has been shown to be quickly cleared by endothelial cells of the liver following injection into mice (25, 26). We have used DiI-Ac-LDL as an aid in histological identification of endothelial cells in frozen sections of murine liver following intravenous injection of 10 μg/ml DiI-Ac-LDL for 4 h at 37°C. Mixed cultures of BAECs and BASMCs (a and b), and BAECs and BRPs (c and d) are shown. e and f are of a primary adrenocorticotoid microvessel culture. In all cases, only cells that appear to be endothelial are labeled with the Dil-Ac-LDL. Bar, 20 μm. × 250.
injection of the lipoprotein. We have also used the fluorescent lipoprotein to aid in identification of capillary fragments in collagenase-digested tissues. Preliminary studies indicate that Dil-Ac-LDL can be used to label capillary endothelial cells in the chick chorioallantoic membrane. The techniques reported here could also be used to quickly obtain endothelial cells in cases where large numbers of endothelial cells are needed in short times, such as to serve as a lining for vascular grafts (27, 28).

In summary, we have demonstrated that labeling endothelial cells with Dil-Ac-LDL is an improved method for specifically visualizing endothelial cells without affecting cell viability. Utilizing this labeling procedure in conjunction with cell sorting technology, we have increased the speed and efficiency of techniques used for the isolation of a pure cultures of microvascular endothelial cells.

* Netland, P. A., J. C. Voyta, and B. R. Zetter, unpublished results.

The authors thank Dr. Judah Folkman for his continuous support and encouragement throughout these studies and Dr. Patricia D’Amore for her critical review of the manuscript. We also acknowledge Dr. Marc Lalande and Mr. Robert Hoffman for their assistance with the cell sorting and Dr. Alicia Orilidge for suggesting that we add heparin to our primary cultures. Bonnie Troped provided expert assistance with preparation of the manuscript and Annette Fanslow provided expert technical assistance.

Funding was provided by grants from the Texas Heart Association (D. P. Via), the National Cancer Institute (B. R. Zetter) (R01 CA37393), and the Monsanto Corporation. B. R. Zetter is the recipient of a Faculty Research Award (#263) from the American Cancer Society.

Received for publication 29 May 1984, and in revised form 1 August 1984.

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