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Purification of Murine Endothelial Cell Cultures by Flow Cytometry Using Fluorescein-Labeled Griffonia Simplicifolia Agglutinin

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Griffonia simplicifolia agglutinin (GSA) is a valuable histochemical tool in the identification of endothelium. In this study GSA labeled with fluorescein isothiocyanate (GSA-FITC) was used to purify cultures of murine cerebral microvascular endothelium. Cultures were stained with GSA-FITC, then sorted using a fluorescence-activated cell sorter (FACS). GSA-positive endothelial cells were collected, re-cultured, and subsequently re-analyzed by FACS using GSA-FITC. Cultures that initially contained 80 ± 3 to 89 ± 3% (X ± SE) GSA-positive cells were purified to 98 ± 1% positivity. Immunohistochemistry with an anti-muscle-actin antibody confirmed that FACS sorting of GSA-FITC-stained cells effectively removed contaminating smooth muscle cells from endothelial cell cultures. Viability, proliferation, and prostaglandin production of the cells was unaltered by lectin staining and FACS sorting. Thus, GSA-FITC can be used in conjunction with flow cytometry to enhance the purity of murine endothelial cell cultures without adversely affecting cell viability, growth, or metabolism. (Am J Pathol 1989, 134:1227–1232)

Griffonia (formerly Bandeiraea) simplicifolia agglutinin (GSA) is a lectin with high affinity for α-D-galactopyranosyl residues.[1,2] The binding of GSA to carbohydrate moieties on cell surfaces has been demonstrated by our laboratory and other investigators to be a specific histochemical marker for endothelial cells from selected organs and tissues of certain species.[3–8] In the brain, the isolectin GSA-IB_4 has been shown to bind to endothelium, providing a histochemical method for distinguishing endothelium from neurons, astrocytes, oligodendroglia, and vascular smooth muscle cells.[6,8] Some ependymal cell binding of GSA also has been noted in mice (personal observation). Previously, we have demonstrated the use of the isolectin GSA-IB_4 labeled with fluorescein isothiocyanate (GSA-FITC) as a probe to monitor murine endothelial cell culture purity.[6,8]

Recent advances in flow cytometry technology have enabled investigators to purify several different types of live cells,[9–14] but few studies have been conducted on endothelial cell cultures. Some have been successful that used fluorescent LDL taken up specifically by endothelium.[15] Still other investigators have reported the use of flow cytometry to separate Kupffer cells from hepatic endothelial cells on the basis of size.[14] Because the GSA lectin has endothelial specificity *in situ* and in culture, and because vascular cell cultures derived from microvessels can contain at least two cell types, we have developed a technique for purifying murine cerebromicrovascular endothelial cell cultures using GSA-FITC binding in conjunction with fluorescence-activated cell sorting.

Materials and Methods

Cell Culture

Cerebral endothelial cell cultures were isolated and maintained as described previously.[16,17] Briefly, the brains of mouse pups were aseptically removed under general anesthesia and disrupted in a Dounce homogenizer. Microvessels were collected either on nylon mesh (150 μ) or glass beads and plated onto plastic tissue culture dishes. Endothelial cells migrating from vessels were pooled to form a line of proliferating endothelia that was maintained in Lewis media (LM) containing 10% fetal calf serum (FCS). The present studies were performed on two Balb/c-derived (MB114 and MB123) and one Swiss-Webster-derived (MBSW-3) lines of endothelium. Identity and purity of cultures were confirmed by light and electron micros-

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copy, thrombomodulin activity, and GSA (Sigma, St. Louis, MO) histochemical staining.[8] All studies were performed on confluent cultures of endothelium between passages 5 and 11.

Histochemistry

GSA-Fluorescein Isothiocyanate

To check the purity of endothelial cell cultures, pre- and postsorted cells were grown on Lab-Tek glass chamber slides (Miles, Naperville, IL). At confluence, cells were washed three times with cold phosphate-buffered saline (PBS) containing 0.1 gm/l calcium (pH, 7.4). Cells were then covered with 0.5 μg/ml GSA-FITC in PBS/CA^{2+} on ice for 30 minutes. As a control, 0.2 M α-D-galactose (Sigma) was added to the GSA-FITC before staining cells to selectively block the binding of GSA to the cell surface. All cells were then washed three times in PBS, coverslipped, and viewed under a fluorescence microscope.

Muscle-Specific Actin Antibody

To check for smooth muscle cell contamination, cells grown on glass slides were fixed in cold methanol, washed with PBS (pH, 7.4), and incubated in PBS + 20% FCS for 30 minutes at 37 C. Cells were again washed with PBS, then covered with mouse monoclonal anti-α-actin antibodies (HUC-13 provided by James Lessard, Cincinnati, OH[18] and HHF-35 provided by Allen Gown, Seattle, WA[19]; both are IgG antibodies) and allowed to incubate at 37 C for 60 minutes. After another wash in PBS, the cells were serially incubated with biotinylated rabbit anti-mouse-IgG antibody and avidin-biotin-peroxidase complex (DAKO, Santa Barbara, CA). Anti-α-actin antibody binding was visualized by adding a 0.2 mg/ml diaminobenzidine solution that contained 20 μl of 1% H_2O_2 and incubating at 37 C for 20 minutes. After this, cells were washed with PBS, counterstained with hematoxylin, and coverslipped.

Glial Fibrillary Acidic Protein (GFAP) Antibody

To check for astrocyte contamination, cells grown on glass slides were fixed in cold methanol. After PBS washes, cells were incubated with PBS supplemented with 20% FCS for 30 minutes at 37 C. Cells were again washed with PBS before 2.5 ng/ml polyclonal rabbit anti-GFAP-antibody[20] (DAKO) was added to the cells for 120 minutes at 37 C. After a PBS wash, fluorescein-conjugated goat anti-rabbit IgG (DAKO) was added for 30 minutes at 37 C. Finally, slides were washed with PBS and coverslipped.

Flow Cytometry

Confluent cells were removed from culture flasks using 0.25% trypsin + 0.1% EDTA for 5 minutes at 37 C and suspended in LM supplemented with 20% FCS. Cells were then pelleted and resuspended in PBS + Ca^{2+} supplemented with 10% FCS at a final cell density of 10^6 cells/ml. GSA-FITC was added to a final concentration of 20 μg/ml and cells were incubated on ice for 30 minutes. As controls, some tubes received either no lectin or lectin preincubated with 0.2 M galactose. All cell samples were then pelleted and washed three times with cold PBS supplemented with 10% FCS. Cells were resuspended in LM supplemented with 20% FCS in sterile sorting tubes and kept on ice. Each sorting tube received 10 μl of 0.05 mg/ml propidium iodide in PBS immediately before sorting on a fluorescence-activated cell sorter (FACS 440; Becton Dickinson Co., Mountain View, CA). Background was eliminated by establishing gates to monitor live cells only and not debris. Unstained cells served as controls. GSA-FITC-positive cells were collected (10^5 for each tube), immediately pelleted, resuspended in LM supplemented with 20% FCS, and plated into 60-mm petri dishes. Analysis of the FACS data was done by Electric Desk (Version 1.7) on a VAX computer.

Cell Proliferation Experiments

To assess cell proliferative capacity in the postsorted cultures, both pre- and post-sorted cells were seeded at 10^5 cells/well onto 96-well culture plates in LM supplemented with 10% FCS. Cells were incubated at 37 C, 5% CO_2 for 72 hours, then pulsed with 1 μCi/well of ^{3}H-thymidine (Amersham; Arlington Heights, IL) for 24 hours. Cell nuclei were then harvested onto glass fiber filters using a Mini-Mash II (MA Bioproducts; Walkersville, MD). Radioactivity in filter disks was determined by liquid scintillation.

Pre- and post-sorted cell cultures were also seeded onto 24-well plates at 50,000 cells/well in 1 ml of LM supplemented with 10% FCS and allowed to adhere overnight at 37 C in 5% CO_2. On the following day, cells counts were determined in triplicate on trypsinized cells using a Coulter counter (Coulter Electronic, Hialeah, FL). Cell counts were subsequently determined every 2 days up to day 11 after seeding. Media was changed every 2 days.

Prostaglandin Synthesis

To assess the metabolic function of endothelial cells before and after FACS sorting, cells were grown to confluence and stimulated with 7.5 μM [^{3}H]-arachidonic acid. After a 1-hour incubation, the media was collected and

centrifuged to remove cellular debris. Media lipids were extracted with a 2:1 (vol/vol) mixture of chloroform:methanol containing 1% acetic acid. Extracted eicosanoids were analyzed by high-performance liquid chromatography (HPLC) on a Beckman 322 HPLC gradient system equipped with a C18 reverse phase column containing 5 μ spherical packing, using a gradient of water adjusted to pH 3.5 with phosphoric acid and acetonitrile, as described previously.[21] Radioactive products of ^{3}H-arachidonic acid were compared with the following standards: 6-ketoprostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), $PGF_{2\alpha}$, PGE_2, 12-hydroxyeicosatetraenoic acid (12-HETE), 5-HETE, 15-HETE, hydroxyheptadecatrienoic acid, leukotriene B_4, and arachidonic acid.

Results

Flow Cytometry

Presorted cells that were GSA-FITC positive exhibited a significant upward shift in fluorescence intensity compared with unstained controls (Figure 1); however, the curves for stained cells and unstained controls overlapped, indicating the presence of GSA-negative cells in these endothelial cell cultures. Of the three cell lines examined, MBSW-3 possessed the highest percentage of nonstaining cells, followed by MB114 and MB123, respectively (Table 1). Cells from all three lines that were incubated with α-D-galactose along with GSA-FITC exhibited fluorescence similar to unstained controls (data not shown). When post-sorted cells were examined, all three cell lines had reduced percentages of nonstaining cells compared with unstained controls as measured by fluorescence intensity on the FACS (Table 1; Figure 1). Propidium iodide incorporation of pre- and postsorted cells was similar, indicating a minimal percentage of only 1% dead cells.

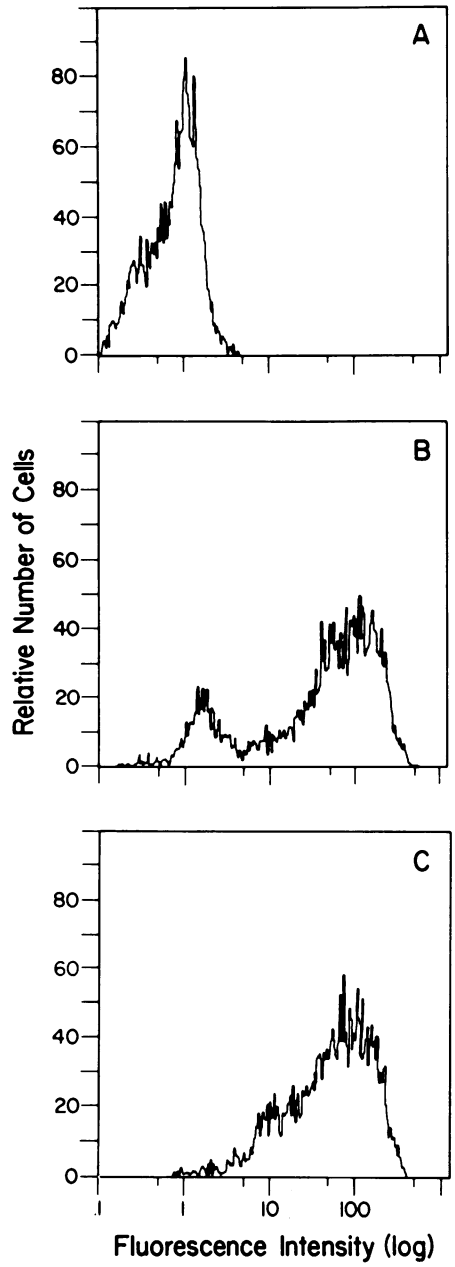

Figure 1. *Flow cytometric analysis of pre- and postsorted MB123 cells using GSA-FITC.* A: *Presorted unstained cells.* B: *Presorted cells stained with GSA-FITC.* C: *Postsorted cells stained with GSA-FITC.*

Histochemistry

Pre- and postsorted endothelial cells that were stained with GSA-FITC exhibited high levels of fluorescence staining. GSA-FITC staining was confined primarily to the surface membrane (Figure 2A). Before sorting, all cell lines contained anti-α-actin positive smooth muscle cells (Figure 2B). A significant reduction in the contamination of smooth muscle cells was noted after sorting compared with presorted controls (Table 1). Endothelium and smooth muscle cells are morphologically distinct and no cross-over staining (GSA staining smooth muscle cells or anti-α-actin antibodies staining endothelium) has been observed. Rare α-actin-positive cells may, therefore, be retained in the postsorted endothelial cultures by adhering to endothelial cells during the FACS processing.

Cell Proliferation

Cell lines that had been treated with GSA-FITC and sorted on the FACS displayed a growth rate by ^{3}H-thymidine incorporation similar to presorted controls. Three days after plating, ^{3}H-thymidine incorporation by MB123 pre- and

Table 1. *FACS and Immunohistochemical Analysis of Pre- and Postsorted Cell Lines*

Endothelium	Passage	FACS GSA	Histochemical staining	
			α-actin	GFAP
Presorted cells				
MBSW-3	5–10	80 ± 3*	++	ND
MB114	5–10	88 ± 4	+	ND
MB123	5–10	89 ± 3	+	ND
Postsorted cells				
MBSW-3	6–11	97 ± 1	±	ND
MB114	6–11	97 ± 2	±	ND
MB123	6–11	98 ± 1	±	ND

* Values are means ± SE of triplicate determinations on the FACS.
++, Several positive cells were noted.
+, Few positive cells were noted.
±, Rare positive cells were noted.
ND, No positive cells were demonstrated (none demonstrated).

postsorted cells was 19,240 ± 840 CPM/well and 18,390 ± 830 CPM/well, respectively (Figure 3). In addition, pre- and postsorted MB123 cells displayed nearly identical growth curves (Figure 4). Similar results were obtained with the other endothelial cell lines.

Prostaglandin Production

Analysis of endothelial cell cultures revealed no significant difference in prostaglandin production between pre- and postsorted cells. The cell line MB123 converts arachidonic acid predominately to PGI_2, PGE_2, and $PGF_{2\alpha}$.[21] Analysis of pre- and postsorted MB123 cells by HPLC revealed qualitatively and quantitatively similar prostanoid profiles of 6-keto-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$. The chromatograms (not shown) were nearly identical to previously published chromatograms.[21]

Discussion

The current study shows the feasibility of using GSA-FITC lectin in conjunction with flow cytometry to purify murine cerebromicrovascular endothelial cell cultures. Previous work in our laboratory and by others has demonstrated the utility of GSA-FITC in identifying cerebral endothelium of mice and dogs and characterizing cultures of these cells.[4,6,8] Given the specificity that GSA has for vascular endothelium, there is a great, though previously unexamined potential for using this lectin in combination with the FACS for purifying endothelial cell cultures. One reason for the lack of prior success with lectins is the intrinsic problem of lectin-induced agglutination of cells.[12,13] We have circumvented this problem by using a subagglutinating concentration of GSA, ie, 20 μg/ml, which is in agreement with previously published data.[12] Another intrinsic

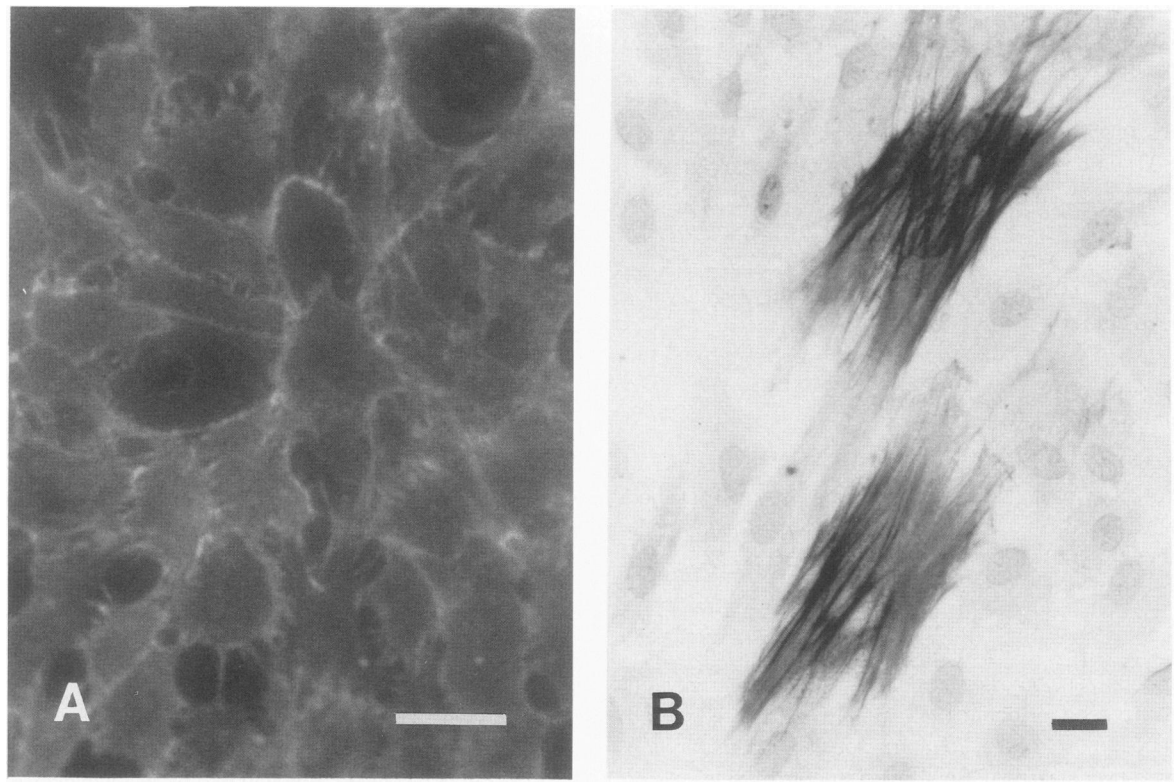

Figure 2. *GSA-FITC histochemistry and α-actin immunohistochemistry.* **A:** *Presorting endothelial cell culture stained with GSA-FITC (×800 final magnification; bar = 20 μ).* **B:** *Presorting endothelial cell culture stained with anti-α-actin antibody HHF-35 using an avidin-biotin-peroxidase technique (×400 final magnification; bar = 20 μ).*

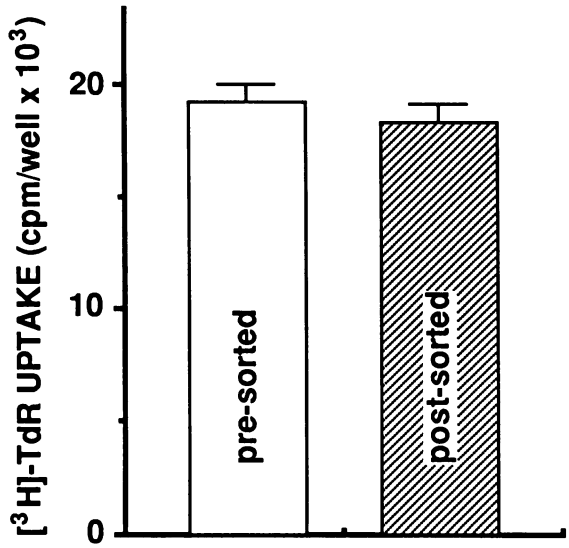

Figure 3. *^{3}H-thymidine incorporation by pre- and postsorted cerebral endothelial cell cultures. ^{3}H-thymidine incorporation during a 24-hour incubation was determined on day 3 after plating the cells. Data points are the X ± SE of triplicate experiments.*

problem for the use of many lectins in sorting live cells is their extreme toxicity; Ricinus communis agglutinins are especially toxic.[22] The present studies demonstrate that toxicity is, however, not a factor in the use of GSA (*vide infra*).

In the past, a significant problem in the purification and characterization of cerebromicrovascular endothelium has been the lack of reagents that bind specifically to the endothelial cell surface.[23] Until now, immunohistochemistry of Factor VIII associated antigen (von Willebrand factor) has been used to identify vascular endothelium, but because of the intracytoplasmic location of the antigen[24,25] it has not been useful for flow cytometry of live cells. In the case of murine cerebromicrovascular endothelium, no Factor VIII associated antigen is present in passaged cells, precluding its usefulness in purifying these cells. Other endothelial markers such as thrombomodulin antigen and Ulex europeaus lectin have been demonstrated to be excellent histologic markers in certain species,[24-28] but their role in flow cytometry has not been demonstrated. The present study corroborates previous findings that the GSA lectin is highly specific for murine endothelial cells. Other investigators have reported that GSA may bind to sites on the basement membrane,[1] to sites predominately confined to the surface membrane,[5,7] or to intracytoplasmic organelles.[4] Our results indicate that the GSA binding sites appear to be located on the surface membrane (Figure 2). Due to the location of at least some binding sites on the cell surface, we have been able to extend the use of GSA from identifying endothelium to purifying live endothelial cell cultures.

No previous studies have documented the effect of GSA on cell viability, growth, and metabolism. Our data indicate that GSA-FITC staining and FACS processing do not affect cell viability, growth rate, or metabolic potential. They further suggest that these cultured endothelia do not rely on the contaminating smooth muscle cells for major cell functions. We cannot, however, exclude the possibility that GSA staining, cell sorting, and/or removal of smooth muscle may alter some untested biologic properties of these cerebral endothelia.

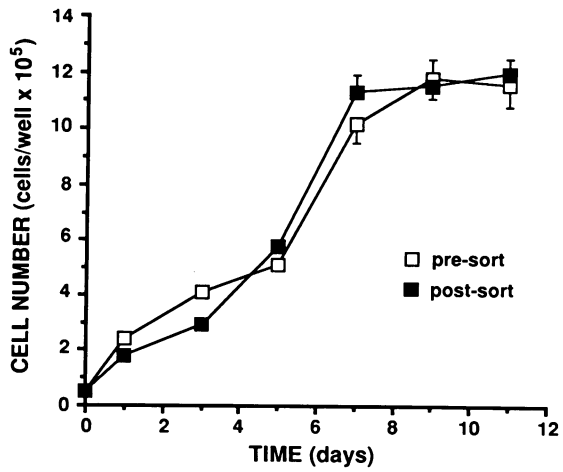

Figure 4. *Growth curves of pre- and postsorted cerebral endothelial cell cultures. Cells were plated on day 0 at a density of 50,000 cells/well. Cells from triplicate wells were trypsinized and counted on days 1, 3, 5, 7, 9, and 11. Data points are the X ± SE of triplicate wells.*

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