

Migrating Endothelial Cells Are Distinctly Hyperglycosylated and Express Specific Migration-associated Cell Surface Glycoproteins

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Abstract. Migration of endothelial cells is one of the first cellular responses in the cascade of events that leads to reendothelialization of an injured vessel and neovascularization of growing tissues and tumors. To examine the hypothesis that endothelial cells express a specific migration-associated phenotype, we analyzed the cell surface glycoprotein expression of migrating bovine aortic endothelial cell (BAECs). Light microscopic analysis revealed an upregulation of binding sites for the lectins Concanavalin A (Con A), wheat germ agglutinin (WGA), and peanut agglutinin after neuraminidase treatment (N-PNA) on migrating endothelial cells relative to contact-inhibited cells. These findings were confirmed and quantitated with an enzyme-linked lectin assay (ELLA) of circularly scraped BAEC monolayers. The expression of migration-associated cell surface glycoproteins was also analyzed by SDS-PAGE. The overall expression of cell surface glycoproteins was upregulated on migrating BAECs. Migrating BAECs expressed Con A- and WGA-binding glycoproteins with apparent

molecular masses of 25 and 48 kD that were not expressed by contact-inhibited BAEC monolayers and, accordingly, disappeared as circularly scraped monolayers reached confluence. Subconfluent BAEC monolayers expressed the same cell surface glycoconjugate pattern as migrating endothelial cells. FACS analysis of circularly scraped BAEC monolayers showed that the phenotypic changes of cell surface glycoprotein expression after release from growth arrest occurred before the recruitment of the cells into the cell cycle (3 vs. 12 h). Suramin, which inhibits endothelial cell migration, abrogated the expression of the migration-associated phenotype and induced the expression of a prominent 28-kD Con A- and WGA-binding cell surface glycoprotein. These results indicate that endothelial cells express a specific migration-associated phenotype, which is characterized by the upregulation of distinct cellular glycoconjugates and the expression of specific migration-associated cell surface glycoproteins.

REENDOThelialization of injured blood vessels and neovascularization of tissues in embryonic development, wound healing, and neoplasia proceed in highly ordered cascades of events that both begin with endothelial cell migration (Madri et al., 1991; Noden, 1991; Lindner et al., 1989; Folkman, 1985). Endothelial cells migrate out from preexisting, contact-inhibited endothelial cell monolayers, either in a sheet-like fashion to cover a denuded area of endothelium (Madri et al., 1991; Lindner et al., 1989) or in sprouts of new capillaries to provide vascular perfusion to a growing tissue (Ausprunk and Folkman, 1977). Although endothelial cell migration in reendothelialization and neovascularization is distinct with respect to initiators (injury vs angiogenic factor), affected vessel caliber (large vessels vs capillaries), and functional purpose (repair vs new blood vessel formation), the molecular events that regulate migration may be similar. Accordingly, in vitro

studies on endothelial cell migration have most often been conducted with endothelial cells derived from large vessel origin (e.g., bovine aorta), using either mechanically injured monolayers or sprouting "capillary" bovine aortic endothelial cell (BAEC)¹ tubes as models (Sholley et al., 1977). Within the last two decades, both the influence of growth factors and the role of distinct components of the extracellular matrix have emerged as potential regulators of endothelial cell migration. For example, the release of endogenous basic fibroblast growth factor (bFGF) or coatings with collagens of types I and IV have been identified as requirements for endothelial cell migration in vitro (Tsuboi et al., 1990; Rosen and Goldberg, 1989; Sato and Rifkin, 1988; Madri et al., 1988; Madri and Stenn, 1982). These studies have given little attention to exploring quantitative or qualitative alterations in distinct cell surface receptors of growth factor and

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1. *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cell; Con A, Concanavalin A; ELLA, enzyme-linked lectin assay; N, after neuraminidase treatment; PNA, peanut agglutinin; RCA-I, *Ricinus communis* agglutinin I; SBA, soybean agglutinin; WGA, wheat germ agglutinin.

matrix mediators of endothelial cell migration. Recent reports, however, indicate that modulation of endothelial cell surface protein expression and organization (e.g., low affinity integrin receptors) often occurs in response to changes in adhesive substratum, soluble factors, migration, and proliferation rate (Lampugnani et al., 1991; Basson et al., 1990; Ingber, 1990).

The present study intends to characterize the migration-associated endothelial cell surface phenotype, focusing on glycoconjugate expression in migrating versus contact-inhibited endothelial cells. This task is accomplished by lectin cytochemistry to analyze individual, migrating endothelial cells and by an enzyme-linked lectin assay (ELLA) to quantify differences in glycoconjugate expression in populations of migrating versus growth-arrested, contact-inhibited endothelial cells. Our data show a consistent upregulation of Concanavalin A (Con A)-, wheat-germ agglutinin (WGA)-, and peanut agglutinin (PNA) after neuraminidase treatment (N-PNA)-binding sites on migrating endothelial cells. Two migration-associated, Con A- and WGA-binding endothelial cell surface glycoproteins of 25 and 48 kD are identified.

Materials and Methods

Cells and Materials

Endothelial cells (BAECs) were isolated from thoracic aortas of 18-mo-old bovines as described by Booyse et al. (1975). Cells were cultured at 37°C in 75-cm² flasks in DME containing 10% heat-inactivated FBS and frozen in liquid nitrogen at passage 2 or 3. Endothelial cells were used within 10 passages for subsequent experiments.

Migration Assays

Two different assays were used to study migration-associated endothelial cell surface glycoconjugate expression. The first assay served to probe individual, migrating BAECs by lectin cytochemistry. BAECs were grown within the confines of rectangular silicon templates (Heraeus, Hanau, Germany) inserted into 6-well cell culture dishes (Falcon, Becton Dickinson, Oxnard, CA) (Augustin-Voss and Pauli, 1992). Upon reaching confluence, BAECs were released from growth arrest by removing the silicon templates. Cells were allowed to migrate for 24 h, after which time they were analyzed by lectin staining. The second assay was a circular scraping technique that was designed to study glycoconjugate expression of migrating cell populations using the ELLA technique. BAECs were grown to confluence in 24-well plates and then circularly scraped with a soft rubber comb. This technique yielded alternate rings of confluent BAECs and bare plastic (Fig. 1). BAEC glycoconjugate expression was analyzed by this method at confluence and various time points after scraping. This method was also used for the analysis of surface iodinated proteins by PAGE but, for this purpose, cells were grown in 100-mm dishes.

Lectin Cytochemistry and Enzyme-linked Lectin Assay (ELLA)

BAECs used for lectin cytochemistry and ELLA analyses were washed three times with PBS containing 1.0 mM CaCl₂ and 1.0 mM MgCl₂ at room temperature, and processed as described (Augustin-Voss et al., 1991). In brief, cells were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 15 min. Fixed cells were washed three times with PBS containing 0.2% gelatin (PBS-G), then incubated with 1 μg/ml of biotinylated lectin (Vector Laboratories, Inc., Burlingame, CA) for 30 min. After thorough washing with PBS-G, BAECs were incubated with 1 μg/ml of streptavidin-HRP (Zymed Laboratories, Inc., South San Francisco, CA) (5 min), and washed again with PBS-G. For ELLA experiments, bound lectin-enzyme complexes were visualized after light protected incubation with 0.4 mg/ml of *o*-phenylenediamine (Sigma Chemical Co., St. Louis, MO) and 12 μl/ml of 1% H₂O₂ in 50 mM citrate-buffered 0.15 M saline (pH 7.6, 75 μl/well, 5 min). The reaction was stopped

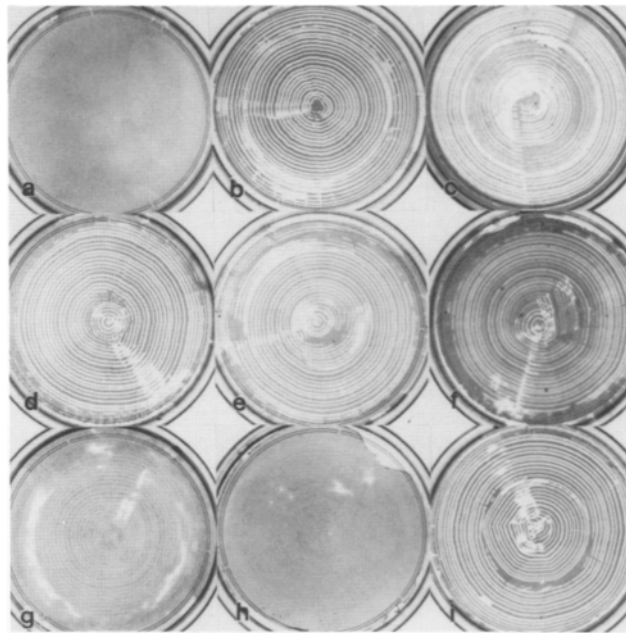


Figure 1. Circular scraping of cultured cell monolayers to study populations of migrating cells. Endothelial cells (BAECs) were grown to confluence (a) upon which time they were circularly scraped with a comb-like instrument (b, immediately after scraping). Circularly scraped monolayers were cultured for different time periods (c, 3; d, 6; e, 12; f, 24; g, 48; and h, 96 h) after which time they were used for the ELLA experiments or radioiodination. (i) Inhibition of endothelial cell migration by 0.5 M suramin added to the culture medium after scraping for 24 h (compare with f).

by addition of 50 μl/well of 1.0 M H₂SO₄ and plates read immediately at 490 nm using a microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). For light microscopic lectin cytochemistry, lectin-enzyme complexes were visualized by incubation with 0.5 mg/ml 3,3'-DAB tetrahydrochloride (Sigma Chemical Co.) and 12 μl/ml 1% hydrogen peroxide in 0.05 M Tris-buffered 0.15 M saline at pH 7.6 (4 min). BAECs were counterstained with Gill's hematoxylin. These procedures yielded selective staining of cell surface glycoconjugates, since fixed BAECs were impermeable to large molecules such as antibodies and lectins, as evidenced by the absence of immunostaining of cytoplasmic factor VIII-related antigen. To account for total cellular glycoconjugate expression, BAECs were also studied under membrane-permeating conditions. Membrane permeation was achieved by incubating fixed BAECs with graded ethanol solutions, followed by a 15-min treatment with 0.3% H₂O₂ in methanol to inactivate endogenous peroxidase. Processing for lectin staining was as described above.

The lectins (and their corresponding sugar specificities) used in this study were Con A (α -Man α -Glc), *Ricinus communis* agglutinin I (RCA-I[Gal β -GalNAc]), WGA (β -GlcNAc β -NeuNAc), PNA (β -Gal-[1-3]-GalNAc β -Gal), and Soybean agglutinin (SBA) (GalNAc β -Gal). The lectin Ulex europaeus agglutinin-I (UEA-I) frequently used as a marker for human endothelial cells does not bind BAECs and was therefore not included in this study. For identification of cryptic PNA- and SBA-binding sites, cells were incubated with neuraminidase (type V, Sigma Chemical Co.) (0.5 U/ml, pH 6.6, 15 min, 37°C) before incubation with the lectin. To evaluate the specificity of the lectin binding, BAECs were stained with lectins that were preincubated with the specific monosaccharide (0.2 M). In all experimental groups, preincubation of lectins with their corresponding monosaccharide resulted in complete inhibition of lectin binding.

Cell Surface Iodination

Endothelial cell surface proteins were labeled by lactoperoxidase-catalyzed iodination, essentially as described by Soule et al. (1982). Briefly, BAECs grown in 100-mm tissue culture dishes (Falcon, Becton Dickinson) were washed extensively in PBS and incubated with 2 ml of 200 mM phosphate buffer, pH 7.3, containing 0.2 mCi Na¹²⁵I (New England Nuclear, DuPont,

Boston, MA), 100 μ l of Enzymobead reagent (Bio-Rad, Richmond, CA), and 100 μ l of 1% β -D-glucose for 25 min at room temperature. The reaction was stopped and unbound iodine removed by washing cells three times with cold PBS. Endothelial cells were collected with a cell scraper, pelleted by centrifugation, and lysed in 50 mM Tris-HCl buffered saline, pH 8.0, containing 1.0% NP-40, 5 mM EDTA, and 0.1% BSA. Nonsoluble material was removed by centrifugation at 15,000 g for 30 min (4°C).

Lectin Affinity Chromatography

To detect specific-binding cell surface glycoproteins, 125 I-labeled cell lysates were precipitated with lectins before SDS-PAGE as follows: A lectin-agarose complex was produced by incubating 20 μ l biotinylated lectin, 1 mg/ml (Vector Laboratories, Inc., Burlingame, CA), 25 μ l avidin-agarose (Vector Laboratories, Inc.), and 55 μ l PBS for 2 h at room temperature. Lectin conjugates were washed 3 times by centrifugation to remove unbound lectin. The lectin-agarose complex was resuspended in PBS and incubated with 125 I-labeled cell lysate for 4 h at 4°C. Lectin-bound glycoproteins were collected after three cycles of washing and centrifugation. The precipitated complex was resuspended in 20 mM Tris-HCl sample buffer containing 10% SDS, 5% 2-mercaptoethanol, and 10% glycerol.

Gel Electrophoresis and Autoradiography

125 I-surface-labeled cell lysates and 125 I-labeled cell surface glycoproteins purified by lectin affinity chromatography were resolved under reducing conditions by SDS-PAGE as described (Laemmli, 1970) using 5–15% discontinuous gels. The dried gels were exposed to Kodak XAR5 film (Eastman Kodak Co., Rochester, NY) at –70°C for 2–7 d.

Flow Cytometry

BAECs of confluent, subconfluent, and circularly scraped monolayers were released from the growth surface with 0.05% trypsin and 0.02% EDTA in HBSS. Cells were collected by centrifugation and resuspended in 0.05 mg/ml of propidium iodide in 0.1% sodium citrate. Flow cytometry of propidium iodide-labeled endothelial cell nuclei was performed using a computer interfaced multiparameter laser flow cytometer (Coulter Epics 752, Coulter Electronics, Hialeah, FL). Laser excitation was by an argon ion laser tuned to 488 nm delivering 100 mW. Data acquisition was performed in the list mode with 10,000 events per file to plot DNA histograms for the determination of cell cycle distribution.

Statistical Analysis

Means of ELLA experiments were compared by two-way analysis of variance. Differences between the experimental groups and the interexperimental absorbance level differences were considered as varying parameters. When a significant difference between the experimental groups was found, individual means were compared by Duncan's multiple range test.

Results

Expression of Lectin-binding Sites on Migrating Endothelial Cells

Using the silicon ring compartmentalization technique, BAECs were released from growth-arrested monolayers and allowed to centrifugally migrate for 24 h. Growth arrested cells and a peripheral rim of migrating cells were then simultaneously probed with a panel of lectins allowing semi-quantitative comparisons of lectin-binding intensities under identical conditions. Con A, WGA, and N-PNA binding intensities were moderately upregulated on migrating cells relative to growth-arrested cells. This difference in lectin binding between migrating and growth-arrested endothelial cells became more prominent when cells were stained under membrane-permeating conditions. Con A staining was particularly intense and was characterized by prominent perinuclear staining (Fig. 2). This staining pattern coincided with

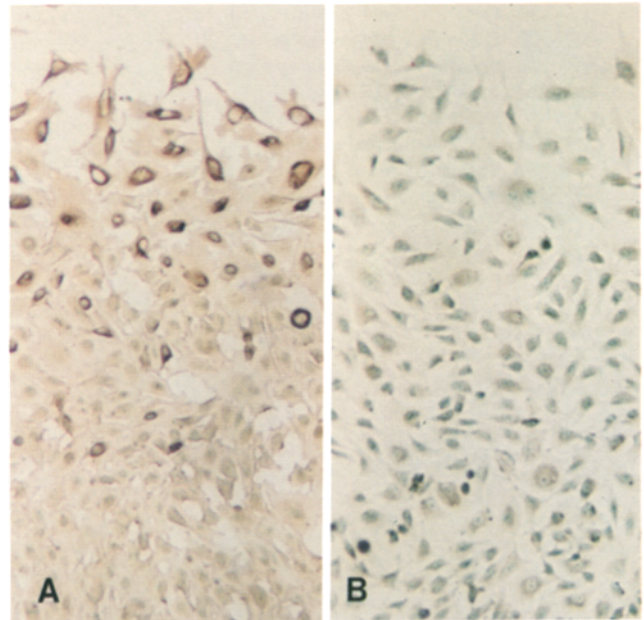


Figure 2. Con A-binding of migrating and growth-arrested endothelial cells. (a) The cells at the migrating rim stain more intensely than the contact-inhibited cells, demonstrating an upregulation of Con A-binding sites on migrating endothelial cells. (b) Preincubation of Con A with methyl- α -D-mannopyranoside completely inhibits Con A-binding.

the perinuclear localization of organelles involved in glycoconjugate synthesis and processing (i.e., Golgi apparatus and ER) and, thus, was indicative of a high rate of synthesis of glycoconjugates with intermediate metabolites that are rich in mannose residues. RCA-I and N-SBA stained both growth-arrested and migrating endothelial cells with similar intensities, whereas PNA and SBA stained neither growth-arrested nor migrating BAECs.

The silicon ring compartmentalization technique was useful in the simultaneous, cytochemical characterization of contact-inhibited and migrating endothelial cells, but was at best semiquantitative and did not allow the analysis of populations of migrating cells. Therefore, populations of migrating endothelial cells were studied in the "circular scraping" assay. After scraping the BAEC monolayers, large numbers of cells simultaneously started to migrate reaching a maximum between 12 and 24 h after which time they gradually became confluent again (Fig. 1). Migrating endothelial cell populations were probed with a panel of lectins and binding intensities were quantitated spectrophotometrically in an ELLA. Confirming the light microscopic lectin cytochemistry data, BAECs strongly bound the lectins Con A, RCA-I, WGA, N-SBA, and N-PNA (Fig. 3). WGA- and N-PNA-binding intensities were significantly upregulated at several time points after circular scraping. Absorbance values of migrating BAECs clearly exceeded the binding intensity of both confluent BAEC monolayers as well as the subendothelial extracellular matrix left on the plastic surface after completely scraping off the BAECs. As monolayers reached confluence, absorbance values decreased again to levels of the original confluent monolayer. It is worth noting that absorbance values were not corrected for total cell number, since confluent and migrating cells covered very different surface

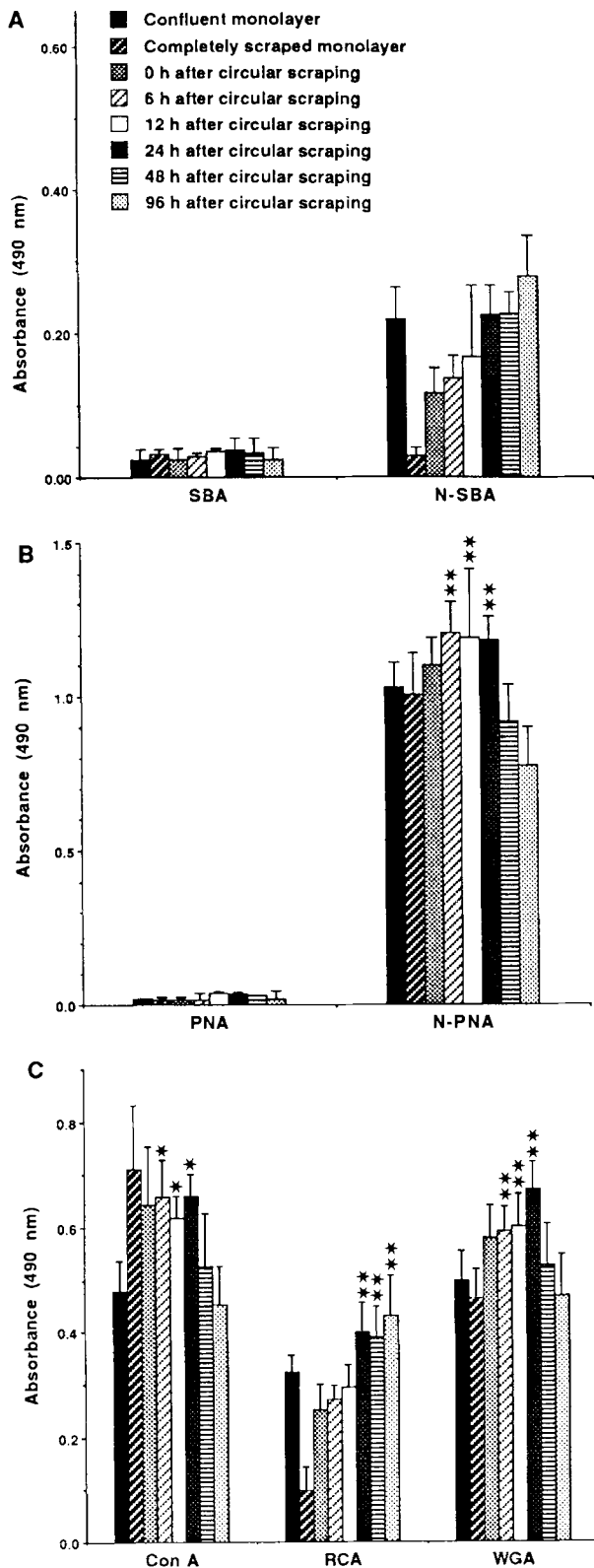


Figure 3. ELLA of migrating endothelial cells. Confluent BAECs were circularly scraped and allowed to migrate for the time periods indicated. Lectin-binding intensities were determined with an ELLA as outlined in Materials and Methods. Cells were probed with SBA (A) and N-SBA, (B) PNA and N-PNA, as well as (C) Con A, RCA-I, and WGA. During migration, binding sites of Con A, WGA, and PNA (after neuraminidase treatment) are upregulated. When scraped monolayer reaches confluence (after 30–

48 h), binding intensities decrease to levels of the original confluent monolayer. The changes of binding intensities of RCA-I and SBA (after neuraminidase treatment) reflect the increases in cell number after circular scraping. Binding of the completely scraped monolayer (second bar) demonstrates lectin-binding of the underlying extracellular matrix, which in the case of Con A quantitatively overshadows the upregulation of lectin receptors on migrating endothelial cells. Results shown here were obtained under permeating conditions. Similar results were obtained under nonpermeating conditions. Absorbance values represent the means \pm SD of three experiments performed in triplicate (*, binding intensity upregulated compared with confluent monolayer, $p < 0.01$; **, binding intensity upregulated compared to confluent monolayer, completely scraped monolayer, and monolayer immediately after scraping, $p < 0.01$).

Thus, considering that fewer BAECs covering a smaller area in migrating cultures stronger bound the lectins WGA and N-PNA than BAECs in confluent cultures, the upregulatory effect can be appreciated even better. The Con A-binding intensity of migrating endothelial cells was upregulated relative to confluent endothelial cells. However, this upregulation could not be reliably attributed to migrating BAECs, since Con A bound stronger to subendothelial matrix than to BAECs. RCA-I- and N-SBA-binding intensities decreased after circular scraping and then gradually increased to the level of the original confluent monolayer as cells grew to confluence. This absorbance profile reflected the surface area covered with cells at different time points after circular scraping and, thus, suggests that RCA-I- and N-SBA-binding sites were not upregulated during endothelial cell migration.

Cell Surface Glycoprotein Expression of Migrating BAECs

Upregulation of WGA- and N-PNA-binding sites on migrating endothelial cells as seen in the circular scraping assay was also observed on subconfluent BAECs. Subconfluent BAEC monolayers had a significantly stronger binding affinity for the lectins WGA and N-PNA than confluent monolayers (Fig. 4). In fact, cells seeded at 1/16 the seeding density of confluent BAECs expressed the same binding intensities as confluent monolayers. In contrast, RCA-I- and N-SBA-binding intensities reflected the difference in the culture dish surface area covered with cells.

BAECs expressed a number of major 125 I-labeled NP-40-soluble cell surface proteins (Fig. 5 A). Most proteins were between 90 and 150 kD, in which autoradiograms showed least resolution. Loading the same amounts of activity, several lower molecular weight bands appeared to be upregulated after circular scraping, but overall cell surface protein expression appeared to be very similar. In contrast, SDS-PAGE of BAEC surface glycoproteins isolated by lectin affinity chromatography revealed distinct differences between growth-arrested and migrating endothelial cells (Fig. 5, B and C). Autoradiograms from migrating endothelial cells consistently showed upregulation of cell surface glycoproteins. As early as 3 h after circular scraping, BAECs expressed new Con A- and WGA-binding glycoproteins with apparent molecular masses of 25 and 48 kD. Expression of these molecules was strongest at 12–24 h after circular scraping after which time the bands disappeared again as monolayers reached confluence. Overexposure of autoradio-

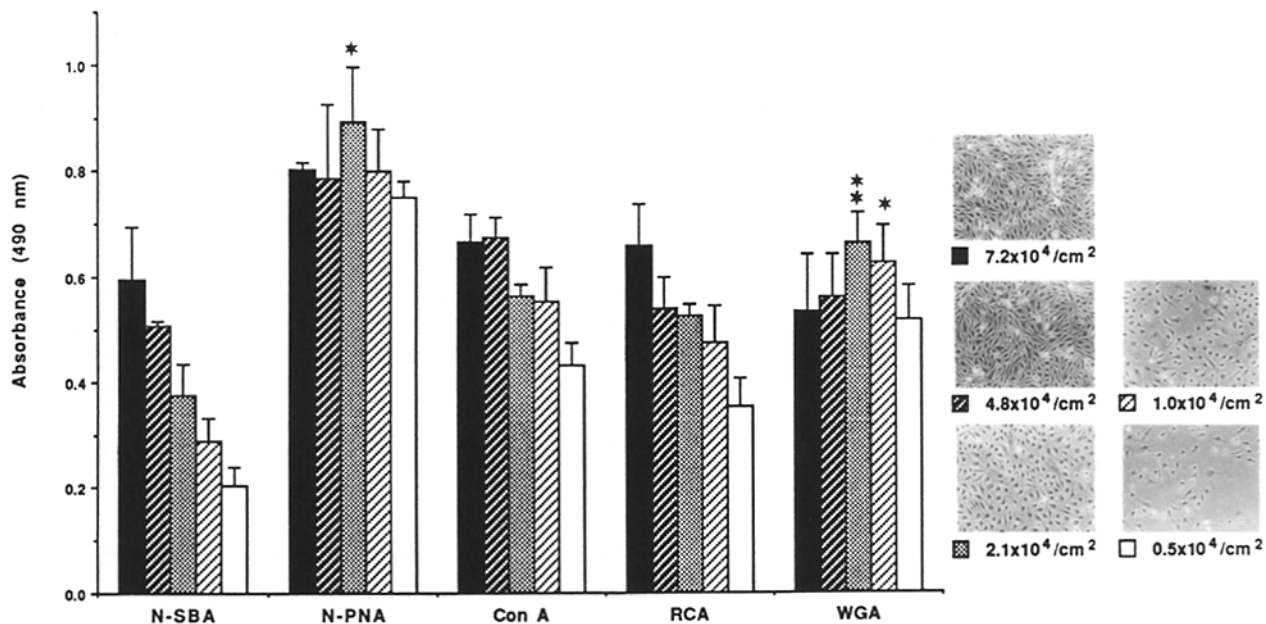


Figure 4. ELLA of confluent and subconfluent endothelial cells. BAECs were seeded at different densities and cultured for 48 h. The figure legend at the right shows cell densities and cell numbers at the end of the culture period. After fixation, cells were probed with N-SBA, N-PNA, Con A, RCA-I, and WGA. Binding sites of WGA and N-PNA are upregulated on subconfluent monolayers. Results are not corrected for cell number, because individual cells in confluent and subconfluent monolayers are very different in size. Thus, the upregulation of lectin-binding sites on subconfluent endothelial cell monolayers can be appreciated, considering that for both, WGA and N-PNA, cells seeded at 1/16 the seeding density of confluent cells still reach the same binding intensities as confluent monolayers. The binding intensities of RCA-I and N-SBA reflect the different cell numbers in the culture dish. Con A binding does not appear upregulated. However, it appears that the decrease of Con A absorbance values is not strictly correlated to the number of cells. Results shown here were obtained under permeating conditions. Similar results were obtained under nonpermeating conditions. Absorbance values represent the means \pm SD of three experiments performed in triplicate (**, $p < 0.01$; *, $p < 0.05$; binding intensity upregulated compared to confluent monolayer).

grams showed background level expression of the 25- and 48-kD glycoproteins in confluent monolayers before circular scraping but they were not detectable in superconfluent monolayers at 96 h after circular scraping (Fig. 5, B and C). The 48-kD cell surface glycoprotein also bound the lectin RCA-I (data not shown).

Analogously to the ELLA experiments, expression of ¹²⁵I-labeled, NP-40-soluble cell surface proteins from confluent BAEC monolayers was compared with that of subconfluent monolayers (Fig. 6 A-C). Using the same amounts of radioactivity for lectin affinity chromatography (Fig. 6 A), total cell surface expression of Con A- and WGA-binding glycoproteins was strongly upregulated on subconfluent monolayers (Fig. 6, B and C). Subconfluent BAEC monolayers also expressed the Con A- and WGA-binding 25- and 48-kD cell surface glycoproteins.

Flow Cytometry of Migrating and Subconfluent BAECs

To verify whether the observed phenotypic changes of migrating and subconfluent BAEC monolayers were attributed exclusively to migration or whether they might in fact reflect proliferation-associated changes, cell cycle distribution analyses of confluent BAECs, BAECs at different time points after circular scraping, and subconfluent BAECs were performed by flow cytometry (Table I). The major recruitment into the cell cycle did not occur before 12 h after circular scraping. In contrast, lectin-binding patterns significantly changed as early as 3-6 h after circular scraping, indicating that the expression of the migration-associated phenotype

preceded proliferation. Interestingly, even the superconfluent monolayers at 96 h after circular scraping were only 90.6% GI arrested which we believe reflected the autocrine activity of early passage BAECs (all experiments were performed with BAECs up to passage 10).

Effect of Suramin on BAEC Surface Glycoprotein Expression

The polyanionic growth factor receptor antagonist suramin is known to inhibit autocrinely regulated endothelial cell migration (Tsuboi et al., 1990) as well as tumor cell-induced endothelial cell migration (Augustin-Voss and Pauli, 1992). Adding 0.5 mM suramin to circularly scraped BAEC monolayers inhibited endothelial cell migration (Fig. 1) and, thus, abrogated expression of the described migration-associated endothelial cell surface phenotype (Fig. 7). Instead, suramin-treated BAECs expressed a prominent 28-kD Con A- and WGA-binding cell surface glycoprotein (Fig. 7). The expression of this molecule was not dependent upon circular scraping, and was also seen in confluent suramin-treated monolayers (data not shown).

Discussion

Endothelial cells in vivo form a confluent, growth-arrested monolayer with a very low turnover rate (Fajardo, 1989). Denudation injury (Lindner et al., 1989) or angiogenic stimulation (Folkman and Klagsbrun, 1987), however, rapidly

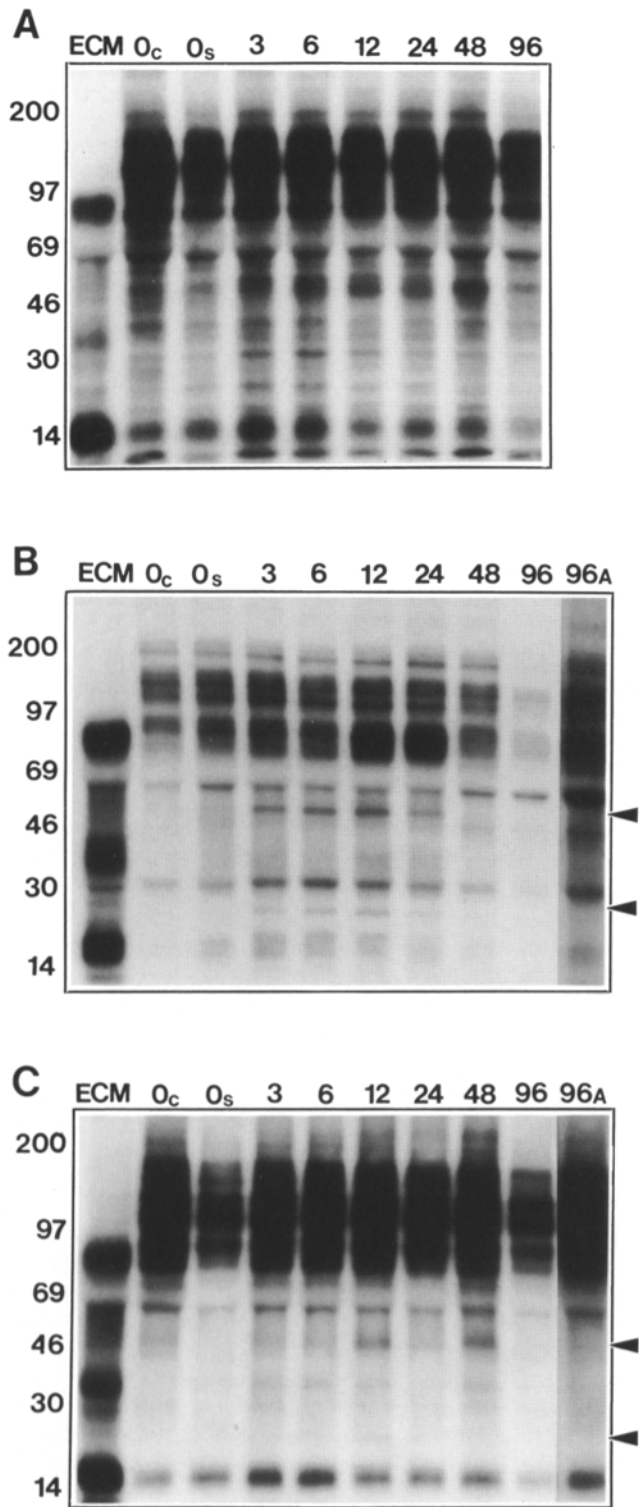


Figure 5. Autoradiogram of cell surface glycoproteins from migrating endothelial cells separated by SDS-PAGE. Confluent BAECs were circularly scraped and allowed to migrate for the time periods indicated (0, 3, 6, 12, 24, 48, and 96 h). Cell surface proteins were iodinated and separated by SDS-PAGE (A). Alternatively, ¹²⁵I-labeled lectin-binding glycoproteins were isolated by lectin affinity chromatography and separated by SDS-PAGE (B, Con A-binding glycoproteins; C, WGA-binding glycoproteins). ECM, Completely scraped monolayer (i.e., labeling of empty dish) at 0 h (control to differentiate cell surface labeling from extracellular matrix labeling in scraped dishes); O_c, confluent monolayer at 0 h; O_s, confluent

induces endothelial cell migration as one of the first steps in a cascade of events that leads to either reendothelialization of an injured vessel or growth of new capillary blood vessels (angiogenesis). Despite enormous efforts to elucidate the regulatory mechanisms of endothelial cell migration, studies that have attempted to characterize the phenotype of migratory endothelial cells are rather limited. They have yielded a migration-associated upregulation in the synthesis of chondroitin sulfate and dermatan sulfate proteoglycans (Kinsella and Wight, 1986) and, more recently, a transient expression of syndecan cell surface proteoglycan in endothelial cells from granulation tissue (Elenius et al., 1991). In an attempt to further characterize the glycoconjugate phenotype of migratory endothelial cells, we report here that endothelial cell migration is associated with distinct surface hyperglycosylation and expression of specific cell surface glycoproteins.

Upregulation of Lectin-binding Sites on Migrating Endothelial Cells

Lectins have been used in the phenotypic characterization of various cell types including endothelial cells (Porter et al., 1991; Schnitzer et al., 1990; Simionescu et al., 1982). Their endothelial cell binding sites are often species- (Holthöfer, 1983), age- (Mills and Haworth, 1986), organ- (Ponder and Wilkinson, 1983), and vascular bed-specific (Soda and Ravassol, 1983; Mills and Haworth, 1986). In the present study, a select battery of lectins was used to characterize the surface phenotype of migrating versus growth-arrested, confluent BAEC monolayers. Semiquantitative light microscopic lectin cytochemistry revealed an upregulation of Con A-, WGA-, and N-PNA-binding sites on migrating endothelial cells. Upregulation of these lectin binding sites lasted throughout the reendothelialization phase of circularly scraped BAEC monolayers and ceased when endothelial cell monolayers became confluent. This distinct pattern of upregulated lectin binding sites on migrating endothelial cells is indicative of an overall increase in the expression of cell surface glycoconjugates containing mannose, N-acetylglucosamine, and/or sialic acid, as well as subterminal galactose. Functionally, the upregulation of lectin binding sites on migrating endothelial cells may have a significant influence on the way endothelial cells interact with their immediate environment including other endothelial cells, "blood cells," most notably leukocytes and platelets (Butcher, 1991; Nguyen, M., J. Folkman, and J. Bischoff, 1991. *J. Cell Biol.* 115:69a), growth factors (Sato and Rifkin, 1988; Tsuboi et al., 1990), etc. For example, binding sites for the lectins Con A, LCA, and PSA expressed most strongly on lamellipodia of migrating endothelial cells have been associated with the preferred adherence of monocytes to these sites (De Bono and Green, 1984; Di Corleto and de la Motte, 1985; Ball et al., 1989). Specific endothelial cell surface lectin staining profiles have also been implicated with the binding of tumor

monolayer immediately after circular scraping (0 h); 96A, overexposure of lane 96 h to demonstrate that the expression of specific migration-associated glycoproteins is not a result of the apparent upregulation of lectin-binding glycoproteins expressed by migrating BAECs. The positions of specific migration-associated glycoproteins are marked with arrowheads.

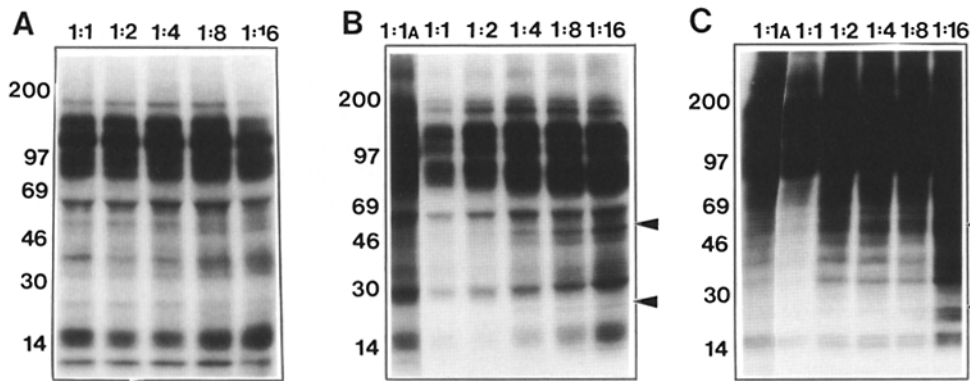


Figure 6. Autoradiogram of cell surface glycoproteins from confluent and subconfluent BAEC monolayers separated by SDS-PAGE. Cell surface proteins were iodinated and separated by SDS-PAGE (A). Alternatively, ^{125}I -labeled lectin-binding glycoproteins were isolated by lectin affinity chromatography and separated by SDS-PAGE (B, Con A-binding glycoproteins; C, WGA-binding glycoproteins). 1:1, 1:2, 1:4, 1:8, and 1:16, different dilutions of endothelial

cells (corresponding to the cell densities and cell numbers shown in Fig. 4). 1:1A, Overexposure of lane 1:1 to demonstrate that the expression of specific glycoproteins is not a result of the apparent upregulation of lectin-binding glycoprotein expression of subconfluent BAECs. The positions of specific migration-associated glycoproteins are marked with arrowheads.

cells that metastasize to specific organ sites (Augustin-Voss et al., 1991).

Migration of Endothelial Cells Versus Proliferation

Migration of endothelial cells during reendothelialization and angiogenesis typically precedes proliferation (Ausprunk and Folkman, 1977; Sholley et al., 1984). Autoradiography of in vitro migrating endothelial cells showed the same phenomenon: Migration is observed as early as 4 h after scraping of a confluent endothelial cell monolayer, yet significant incorporation of ^3H thymidine does not occur until 12–20 h after scraping (Ball et al., 1989; Sholley et al., 1977). In the present study we determined the cell cycle distribution of BAEC after circular scraping to see whether the observed phenotypic changes were migration-associated or whether they reflected cell cycle-dependent, proliferation-associated changes. There was no significant recruitment into the cell cycle for the first 12 h after circular scraping, while the described cell surface phenotypic changes on

migrating BAECs were observed as early as 3–6 h after circular scraping. Furthermore, light microscopic lectin cytochemistry revealed that the phenotypic changes were confined to the cells outgrowing from growth arrest-released monolayers, and were not observed in the zone of proliferation which is located typically behind the migrating front of endothelial cells.

Subconfluent BAECs expressed the same phenotype as migrating endothelial cells. Since these cells are fully recruited into the cell cycle and since migration of endothelial cells precedes proliferation during reendothelialization and angiogenesis, the intriguing question arises whether the migratory phenotype might be essential for the initiation of cell proliferation. A further characterization of proliferation- and tumor-induced endothelial cell proteins recently identified by two-dimensional PAGE (Clarke and West, 1991), might shed new light on possible molecular links between migration and proliferation of cells. Moreover, the demonstrated phenotypic differences between confluent and subconfluent BAEC monolayers illustrate the necessity to carefully monitor the confluence of monolayers used for

Table I. Flow Cytometric Analysis of Cell Cycle Distribution of Confluent Migrating, and Subconfluent BAEC Monolayers

BAEC population	Percent of cells in G1	Percent of cells in GII, S, and M
0 _c	84.5 ± 6.8	15.5 ± 6.8
0 _s	84.1 ± 6.3	15.9 ± 6.3
3 h	82.6 ± 6.5	17.4 ± 6.5
6 h	81.4 ± 5.5	18.6 ± 5.5
12 h	74.6 ± 14.1	25.4 ± 14.1
24 h	73.3 ± 8.3	26.7 ± 8.3
48 h	86.6 ± 2.5	13.2 ± 8.3
96 h	90.6 ± 3.1	9.4 ± 3.1
1:1	81.2 ± 4.8	18.8 ± 4.8
1:2	81.0 ± 3.5	19.0 ± 3.5
1:4	69.3 ± 3.6	30.7 ± 3.6
1:8	61.6 ± 5.8	38.4 ± 5.4
1:16	58.9 ± 1.8	41.1 ± 1.8

Results represent means ± SD of three experiments. 0_c, Confluent monolayer at 0 h; 0_s, confluent monolayer immediately after circular scraping (0 h); 3, 6, 12, 24, 48, and 96 h time periods after release from growth arrest by circular scraping; and 1:1, 1:2, 1:4, 1:8, and 1:16, different dilutions of endothelial cells (corresponding to the cell densities and cell numbers shown in Fig. 4).

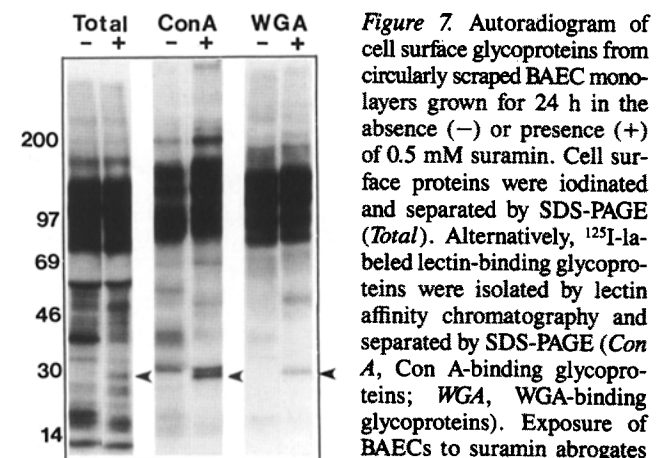


Figure 7. Autoradiogram of cell surface glycoproteins from circularly scraped BAEC monolayers grown for 24 h in the absence (–) or presence (+) of 0.5 mM suramin. Cell surface proteins were iodinated and separated by SDS-PAGE (Total). Alternatively, ^{125}I -labeled lectin-binding glycoproteins were isolated by lectin affinity chromatography and separated by SDS-PAGE (Con A, Con A-binding glycoproteins; WGA, WGA-binding glycoproteins). Exposure of BAECs to suramin abrogates the expression of the specific migration-associated cell surface glycoproteins and induces the expression of a 28-kD Con A- and WGA-binding cell surface glycoprotein (arrowheads).

functional assays involving cell surface molecules, e.g., adhesion assays.

Expression of Specific Migration-associated Glycoproteins

Earlier studies by Vlodavsky et al. (1979) revealed a 60-kD surface protein (CSP-60) that was expressed in subconfluent but not in confluent BAEC monolayers. Although the overall pattern of BAEC surface protein expression observed in that study was similar to the one reported here, we were unable to identify CSP-60 in migrating BAECs, possibly due to the use of a nonionic detergent rather than SDS in the lysis buffer. Instead, we observed two Con A- and WGA-binding glycoproteins of 25 and 48 kD, that were both expressed on migrating and subconfluent BAECs. These glycoproteins were lost when injured BAEC monolayers were fully repaired and assumed a confluent, contact-inhibited monolayer. A potentially interesting link to our detection of specific migration-associated glycoproteins comes from the characterization of hypoxia-induced stress proteins in cultured endothelial cells (Zimmerman et al., 1991; Ogawa et al., 1991). These stress proteins have molecular masses of 34, 36, 47, and 56 kD (Zimmerman et al., 1991) and are thus in a similar molecular weight range as the migration-associated glycoproteins on BAECs reported here. Since hypoxia has been shown to be a triggering mechanism for angiogenesis, it is intriguing to speculate whether these stress proteins are in fact autocrine-regulated activation-associated molecules of endothelial cells. Another potentially interesting endothelial cell-derived molecule is SPARC. This glycoprotein is in the same molecular weight range (43 kD) and is secreted by subconfluent and tube forming endothelial cells but not by confluent endothelial cell monolayers (Iruela-Arispe et al., 1991). However, SPARC known to bind to the leading edges of membranous cell surface extensions if added exogenously (Sage et al., 1989) is a secreted protein that induces a rounded cell morphology and cell proliferation.

Conclusions

Migrating bovine aortic endothelial cells are characterized by a distinct pattern of hyperglycosylation and the expression of specific migration-associated cell surface glycoproteins. The importance of altered glycosylation patterns in cell migration is underscored by the association of distinct carbohydrate moieties with the development of blood vessels in the chorioallantoic membrane (Flynn, E., and D. Ausprunk, 1991. *J. Cell Biol.* 115:402a), and by blocking of capillary tube formation in vitro with an inhibitor of N-linked oligosaccharide processing (deoxymannojirimycin) (Nguyen, M., J. Folkman, and J. Bischoff, 1991. *J. Cell Biol.* 115:69a). The involvement of cell surface glycoconjugates in endothelial cell migration intriguingly parallels the expression of carbohydrate antigens in malignant tumor cells (Miyake and Hakomori, 1991). Neoplastic transformation, tumor cell invasion, and tumor progression have been associated with a variety of structural changes of cell surface carbohydrates, most notably sialylation and β -1-6 linked branching of complex oligosaccharides (Hakomori, 1991; Dennis et al., 1986, 1989). Preliminary studies in our laboratory also show an up-regulation of select lectin binding sites on migratory bovine

fibroblasts, albeit the profile of such lectin binding sites was different between migrating BAECs and fibroblasts (Augustin-Voss, manuscript in preparation). Candidate molecules for the migrating phenotype of endothelial cells (and possibly other cell types) are glycosylated cell-cell and cell-substratum adhesion molecules. Further studies on the identification of these molecules will therefore not only lead to a better molecular understanding of endothelial cell migration during reendothelialization and angiogenesis but might also lead to a new strategy to therapeutically target migratory endothelial cells in situation of pathologic angiogenesis such as tumor neovascularization and diabetic retinopathy (Clarke and West, 1991; McCarthy et al., 1991; Hagemeyer et al., 1986).

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