THE SELECTIVE BINDING AND TRANSMIGRATION OF MONOCYTES THROUGH THE JUNCTIONAL COMPLEXES OF HUMAN ENDOTHELIUM

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The circulating blood monocyte has a high affinity for vascular endothelium. In vitro studies using human endothelium have demonstrated that basal levels of monocyte adherence far exceeds that by neutrophils (8/endothelial cell (EC)[1] vs. 1/EC respectively) (1, 2). What is known about monocyte behavior in vivo is consistent with the higher binding affinity relative to other circulating leukocytes. A large fraction of circulating monocytes reside in marginal pools (3–4) and leave these pools more slowly than do neutrophils (5–6). In an inflammatory focus, diapedesis of monocytes occurs in tandem with neutrophils and persists for longer periods (7). In addition, monocytes adherent to large vessel endothelium have been identified (8–9). These observations are consistent with a basal level of monocyte emigration that maintains the steady-state pool of differentiated tissue macrophages (10).

In this report, we describe an in vitro model of human monocyte-EC interaction. Growth of human umbilical vein EC (HUVEC) on a collagen gel matrix promoted the development of intercellular junctions. These individual EC borders stained with silver salts. The sites of monocyte binding to the EC surfaces as well as their migration could be assessed by light microscopic and electron microscopic methods. Monocytes were found to bind selectively to the intercellular borders of individual EC and migrate at high basal rates between EC without disruption of the EC monolayers.

Materials and Methods

Culture Medium and Serum. EC were routinely cultured in Medium 199 (M199) supplemented with 20% heat-inactivated human serum (HIA HS). M199 was prepared from liquid concentrate using pyrogen-free water, and fresh glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (all from KC Biologicals, Inc., Lenexa, KS) were added. HS was prepared from A+ whole blood drawn from volunteers after informed consent. After clotting (23°C for 1–2 h, 4°C for 2 h), the serum supernate was heat-inactivated (56°C for 30 min), filtered (0.45 μm), and stored at −20°C until used. Previous work showed that the use of HIA AB+ serum for EC culture did not improve EC growth nor did it alter monocyte-EC binding (1). Where specified, the culture medium (M199/20% HIA HS) was supplemented with 150 mg/ml EC

This work was supported by a grant-in-aid from the Squibb Institute for Medical Research and by National Institutes of Health grants HL-27186 and AI-26155. Address correspondence to N. A. Pawlowski; his present address is Division of Allergy and Immunology, The Children's Hospital of Philadelphia, 34th Street and Civic Center Blvd., Philadelphia, PA 19104.

[1] *Abbreviations used in this paper:* EC, endothelial cell; ECGF, EC growth factor; ECM, extracellular matrix; FN, fibronectin; HUVEC, human umbilical vein EC; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

growth factor (ECGF) (Collaborative Research, Waltham, MA) and 75 μg/ml porcine intestinal heparin (Forest Pharmaceutical, St. Louis, MO) (11). These concentrations were determined in separate experiments to yield maximal EC proliferative responses in this system.

Preparation of Collagen and Other EC Growth Substrates. Gels were formed in 16-mm diameter tissue culture wells from bovine collagen (≥99% type I, Vitrogen; Collagen Corp., Palo Alto, CA). One part 10× M199, one part 0.1 N NaOH, and eight parts collagen solution (3 mg/ml) were mixed in sequence and kept at 4°C. Additional base was added to reach a stable pH ≥7.4. Aliquots of 0.5 ml were allowed to gel in each well for 30 min at 37°C, 5% CO. The gels were then incubated overnight with M199 (two changes of 1 ml each) before use in culture. Routinely, the gels were also treated with human fibronectin (FN) (50 μg/ml in normal saline, stabilized with human albumin) (New York Blood Center, New York, NY) for 30 min at 23°C just before their use as culture substrates for EC unless otherwise specified.

Fibrin gels were prepared from a chilled (4°C) mixture containing calcium chloride (10 mM), human thrombin (2 U/ml), and a human fibrinogen (1 mg/ml) (Kabi Diagnostica, Stockholm, Sweden). 0.5-ml aliquots were allowed to gel in 16-mm diameter wells as described for collagen before use as a culture substrate.

Tissue culture grade plastic surfaces were coated with gelatin either derived from swine skin (G2625; Sigma Chemical Co., St. Louis, MO) or of bacteriological grade (BBL Microbiology Systems, Cockeysville, MD) by first incubating culture wells with aliquots (0.5 ml) of 3% gelatin solutions for 2 h at 23°C. The supernatants were aspirated and the dishes were dried at 37°C overnight. Where specified, blank or gelatin-treated surfaces were also incubated with human FN solutions for 30 min at 23°C.

Isolation and Culture of HUVEC. EC were isolated by collagenase (CLSII; Worthington Biochemical Corp., Freehold, NJ) treatment of human umbilical veins as previously described (1). Routinely, unpooled primary cultures were established on FN-treated 35-mm diameter dishes. Cultures containing contaminating monocytes or significant numbers of smooth muscle cells were discarded. At confluence (3–5 d), primary cultures were passaged using 0.01% trypsin (TR3, Worthington Biochemical Corp.) at split ratios of 1–1.5 onto FN-treated collagen gels or other substrates as specified. Culture medium (1 ml/16-mm diameter well) was changed within 18 h of passage and twice weekly thereafter. First passage, pooled HUVEC cultured on collagen reached confluence within 7–10 d and were routinely used within the succeeding 5 d. Immunoperoxidase staining for factor VIII–related antigen (Miles Laboratories Inc., Naperville, IL) was homogeneous in the cultures.

Preparation of Human Blood Monocyte Suspensions. Blood was drawn from healthy human volunteers after informed consent into acid/citrate/dextrose (formulation of Aster and Jandl [12]). Monocytes depleted of platelet contaminants were prepared by a modification (1) of previously described methods (13). The procedure involved the following steps: The platelet-rich plasma was separated by centrifugation at 23°C and discarded; the remaining whole blood cell pellet was washed twice using 2 vol of PBS that lacked calcium and magnesium (PD) (4°C). The mononuclear cell fraction was separated using a conventional density gradient technique using Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). After washing, the mononuclear cells were then subjected to two sequential incubations in fresh human serum containing 5 mM EDTA to remove monocyte-associated platelets (13). While still suspended in serum/EDTA, the mononuclear cells were then fractionated using Nycodenz Monocytes (Nyegaard, Oslo, Norway) (1). The monocyte fraction obtained at the interface was depleted of all granulocytes and contained monocytes in excess of 85% purity (determined by Wright's and peroxidase stains) and >95% viability (trypan blue exclusion). After washing, monocytes were resuspended in M199 at the specified concentration and kept on ice until used (within 30 min of final isolation).

Silver Staining of EC. The method was adapted from that of Poole and Florey (14). Unfixed EC monolayers were washed with three exchanges of 5% glucose solution and then incubated with a solution containing one part freshly prepared 0.25% $AgNO_3$ and five parts 5% glucose. Positive staining became apparent within several minutes of incubation, and optimal development time was determined by inspection under light microscopy. A negative result was retested by exposing a duplicate monolayer to 0.25% $AgNO_3$ for up to 10 min. After washing with 5% glucose, the monolayers were exposed sequentially for 20 s to solutions

of $CoBr_4$ (3%) and NH_4Br (1%), were washed again with 5% glucose, and then fixed with formalin.

For experiments in which the locations of bound monocytes were analyzed by light microscopy, the above procedure was modified. After the appropriate incubation period, the culture supernatants were aspirated and the monolayers were briefly fixed using 2.5% glutaraldehyde in 0.1 M sodium cacodylate/0.1 M sucrose at 23°C for 10 min. The silver staining was then carried out as described above. The monolayers were then returned to the glutaraldehyde fixative and incubated overnight at 4°C. As described previously (15), the silver staining property is lost after fixation with formalin or alcohols. For examination under light microscopy, the collagen disks were removed from the culture wells, shrunken by blotting on an absorbant surface, counterstained with Diff Quick (Dade Diagnostics, Inc., Aguada, PR) to provide nuclear detail of both adherent monocytes and EC, and mounted under glass coverslips.

Electron Microscopy. Samples examined by transmission electron microscopy (TEM) were fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate and 0.1 M sucrose (pH 7.4), washed in 0.1 M sodium cacodylate, postfixed in osmium tetraoxide (1.0%), stained en bloc with uranyl acetate (0.25%), dehydrated in graded ethanol solutions, and embedded in Epon. Thin sections were stained with lead citrate before examination using a Jeol JEM 100CX microscope. For scanning electron microscopy (SEM), samples were fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate containing 0.1 M sucrose (pH 7.4). After washing, samples were dehydrated in ethanol, transferred to amyl acetate, critical point dried in CO_2, and coated with gold before examination under a high resolution Philips SEM 505 scanning electron microscope.

Results

HUVEC Growth on Collagen vs. Other Culture Surfaces. During growth upon a collagen matrix, individual EC developed the capacity to bind metallic silver at their intercellular borders. The resulting continuous, "cobblestone" pattern of staining appeared homogeneous when confluence was well established (Fig. 1, *A–D*). With time in culture (>2 wk), the silver staining pattern became progressively weaker and more uneven as individual EC became larger, multinucleate EC became more numerous, and the level of confluence became increasingly disrupted (not shown). Thus the capacity to bind silver was related to the maturation and senescence of the cultured monolayer.

Primary and first passage HUVEC (pooled or unpooled) cultured upon either blank or FN-treated plastic dishes (FN-plastic) completely lacked this silver-staining property (Fig. 1, *E–F*). However, all such cultures formed monolayers with stainable intercellular junctions when passaged onto a collagen matrix, including mixtures of EC from different individuals. This process was reversible and EC grown on collagen lost their affinity for silver within 24 h after passage back on FN-plastic. Prolonged culture times, up to 20 d, were not sufficient for the staining property to develop in monolayers grown upon FN-plastic. Since HUVEC are isolated from vessels as clumps or sheets, they initially may retain some of their native, intercellular junctional complexes (16). However, the silver reaction product was not detected by light microscopy 12 h after plating freshly isolated HUVEC onto FN-plastic.

Silver staining of EC margins is characteristic of endothelium that has developed upon a native extracellular matrix in vivo (15, 17) and correlates with the development of complex intercellular junctions (15, 18). We therefore examined first passage HUVEC cultured on collagen gels (EC-collagen) in greater detail using TEM. Broad areas of overlap and interdigitation between EC were evident. A total of 20 different intercellular junctional areas from each of 6 separate preparations were

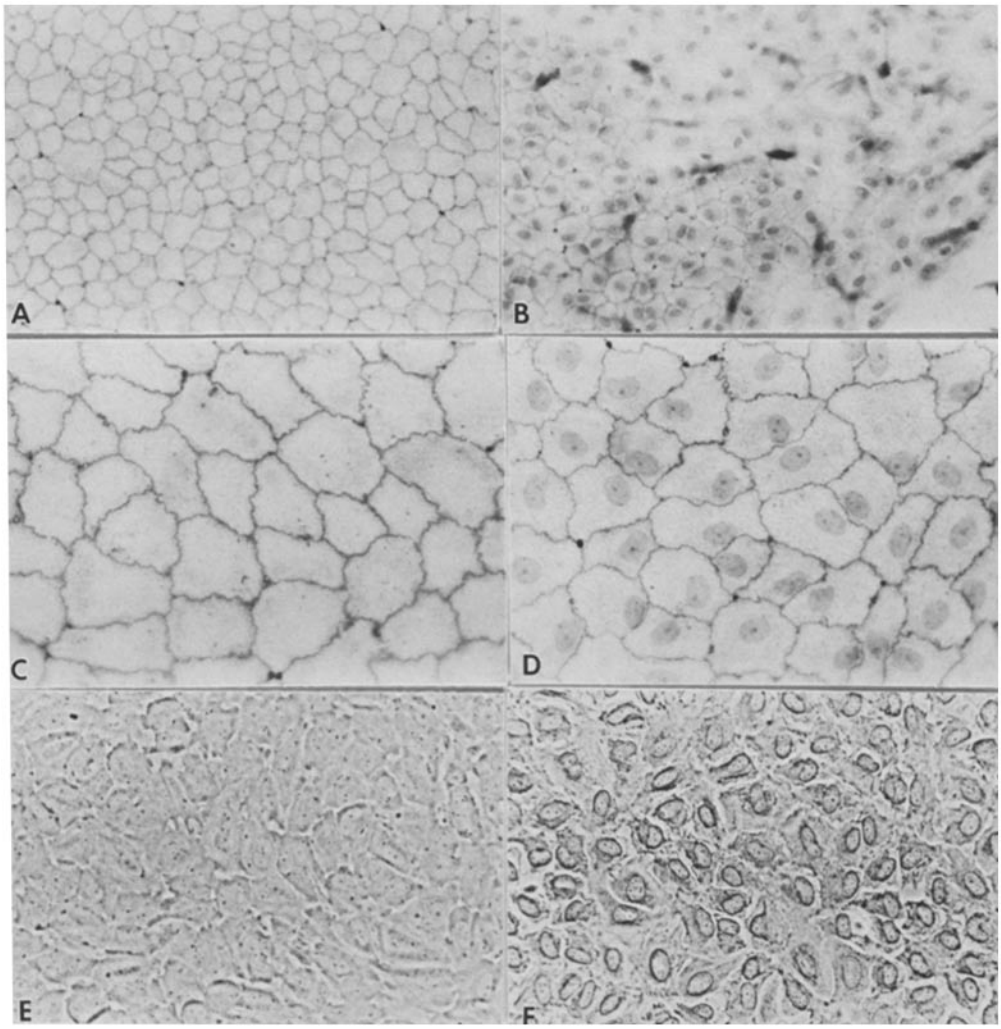

FIGURE 1. Appearance of silver-stained HUVEC monolayers cultured upon a collagen matrix versus plastic. Pooled primary cultures of HUVEC were passaged onto FN-treated collagen gels (*A–D*) or plastic (*E–F*) and cultured in M199/20% HS. (*A*) At confluence, the intracellular borders of EC on collagen stained with silver; ×110. (*B*) EC nuclei are visible after Giemsa staining; ×110. (*C* and *D*) The staining pattern is homogeneous in confluent monolayers, but develops only as confluence is reached in proliferating cultures; ×280. (*E*) HUVEC grown on FN-treated plastic form contact inhibited monolayers as viewed under phase contrast; ×250. (*F*) When these same cultures were exposed to $AgNO_3$, no reaction product formed. The outlines of individual EC and the absence of intercellular connections were evident after fixation and staining with Giemsa; ×250.

examined in detail. In the majority of intercellular areas examined, at least one of two types of complex junctional structures was found. The most common were those that resembled adherens type junctions (19–21) (Fig. 2 *A*). These lacked membrane fusion but contained electron-dense plaques on the cytoplasmic faces of the mem-

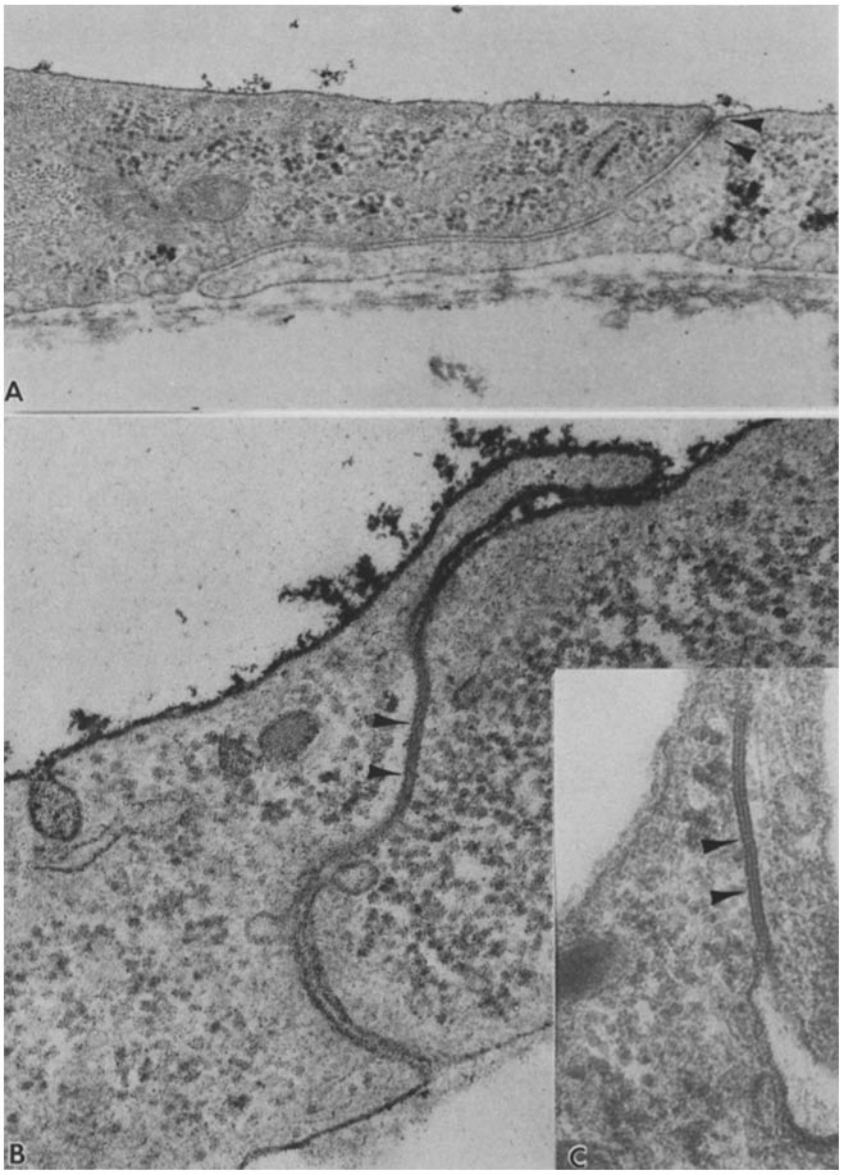

FIGURE 2. Specialized intercellular junctional structures were formed in confluent EC-collagen monolayers. First passage HUVEC were grown to confluence, fixed in glutaraldehyde, and examined by TEM (Materials and Methods). (*A*) Adherens junctions were most frequently identified (*arrowheads*); ×33,000. (*B*) Structures with the characteristics of tight junctions (fusion of outer membrane leaflets) were also common; ×65,000. (*C*) A serial section of the tight junction (cross-section of the membrane) (*arrowheads*); ×65,000.

branes. Sites with membrane fusion resembling tight junctions also were identified (Fig. 2 *B* and *C*).

As outlined in Table I, a variety of other culture conditions did not support the development of silver-stainable intercellular margins. Neither gelatin (± FN) nor a preformed extracellular maxtrix (± FN) was effective. The addition of ECGF plus heparin (11) to the culture medium provided slightly greater proliferative responses but did not alter the silver-staining properties of the EC (Table I).

The collagen gels differed from the other substrates tested (Table I) by their thickness. We therefore examined the properties of HUVEC passaged onto gels of human fibrin of similar thickness (~2 mm). After seeding at near confluent initial densities, HUVEC proliferation was poor on fibrin, even in the presence of ECGF and heparin. However, silver staining of cell borders was found in the confluent areas in EC-fibrin monolayers. These resembled the uneven staining patterns of subconfluent EC-collagen monolayers (Fig. 1 *D*). These results suggest that, although a fibrin matrix does not provide for optimal HUVEC growth and organization (22), the ability to support the development of silver-stainable junctions is not unique to collagen.

Location of Initial Attachment by Human Monocytes to EC Surfaces. In previous studies using HUVEC grown on FN-plastic surfaces, we found that human monocytes bound

TABLE I

Growth Conditions and the Development of Silver-stained Intercellular Junctions

Initial culture surface	Medium containing 20% HIA HS	Supplementation with ECGF and heparin*
Untreated plastic	–	–
FN-coated plastic	–	–
Gelatin‡	–	–
Gelatin‡-FN	–	–
ECM§	–	–
ECM§-FN	–	–
Collagen	+	+
Collagen-FN	+	+
Fibrin	±‖	±
Fibrin-FN	±	±

Primary cultures of HUVEC were pooled and passaged onto individual culture substrates prepared in duplicate as described in Materials and Methods. They were grown to confluence (3 d) using routine medium (M199 containing 20% HIA HS) alone or with added growth factors. At 2 d after confluence, the monolayers were washed and exposed to AgNO3 solution (Materials and Methods) and silver binding was determined by light microscopy. Similar results were obtained in two separate experiments.

* The concentrations of ECGF and heparin (150 and 75 μg/ml, respectively) providing optimal growth enhancement in this system were determined in separate experiments.

‡ Two separate commercial preparations (Materials and Methods) were tested with similar results.

§ Obtained from a commercial source [Extracell dishes (TC-P); Accurate Chemical and Scientific Corp., Westbury, NY].

‖ Monolayers did not reach total confluence and silver stained cell borders were present in only isolated areas of confluence as discussed in Results.

avidly and randomly to human EC (1). The kinetics and maximal capacity for monocyte adherence to EC-collagen were not significantly different (not shown). However, the formation of silver-stained cell junctions provided a "grid" in the plane of the confluent EC monolayer which could be used to directly examine the location of monocyte-EC attachment.

The results of a representative experiment ($n = 3$) are summarized in Table II. Because monocytes can migrate over and through the monolayers, attachment was examined after fixation at the earliest possible time points. Three different methods (described in Table II) were used to establish monocyte-EC contact. Using either technique A (sedimentation by gravity at 4°C) or B (centrifugation on at 4°C), both monocyte binding (306 vs. 398/field) and migration (25 vs. 34% penetrating the monolayer) were comparable at the longest time point studied. However, no monocytes were found penetrating the monolayer at the earliest time point examined (5 min), and <9% had begun to pass through the endothelium by 10 min. Overall these data strongly suggest that very little if any monocyte translocation over the EC surfaces had occurred during the first 5 min of incubation. Therefore the monocyte locations identified at the 5-min time point likely represent the initial sites of monocyte attachment to the EC monolayer. At this time, >77% of the bound monocytes were in direct contact with the silver-stained EC margins, while an additional 9–16% were located within one monocyte diameter away from the silver lines (Table II). <7% of the monocytes bound in the "center" of the EC (greater than one monocyte diameter away from the EC border).

This rate of binding to the "centers" was at least 2.5-fold lower than would be expected if monocyte binding occurred in a completely random manner. This was tested by preparing separate tracings of fields of bound monocytes and silver stain patterns redrawn from actual photomicrographs. Similar analyses of monocyte positions were then made after multiple random placements of the monocytes over the EC outlines. The distribution of monocytes on an EC-collagen monolayer after 5 min of incubation is shown in Fig. 3 *A*. Although an analysis of attachment sites was much more difficult without a "grid" formed by silver staining, monocyte binding to individual HUVEC grown on FN-plastic surfaces appeared random as has been suggested by others (23).

Migration of Monocytes through EC-Collagen Monolayers. Using the "grid" formed in the plane of the EC monolayer by the silver reaction product, several details of monocyte migration through the EC monolayer were apparent under light microscopy (Fig. 3). Monocyte penetration of the EC monolayer occurred only at the intercellular margins of EC (Fig. 3 *B*). The silver reaction product remained visible during all stages of monocyte passage. It encircled monocytes while in the process of transendothelial passage (Fig. 3 *B*), and continuous lines of silver stain were present after the completion of monocyte passage (Fig. 3, *C* and *D*). Finally, even at high monocyte-EC ratios (5:1), nearly all monocytes were found to migrate through the EC monolayer by 60 min of incubation (Fig. 3 *D*). This occurred in the absence of added chemoattractants. In preliminary studies, similar results were obtained with human neutrophils. Although their migration rates were approximately two times faster, neutrophils also penetrated the EC at high baseline levels and failed to disrupt the continuity of the silver-stained EC borders.

The sequence of monocyte binding and migration through the EC-collagen mono-

TABLE II

Location of Monocyte Binding to Individual EC

Technique	Prior treatment	Incubation time at 37°C	Average number of cells/ 40× field	Location of monocytes (●) on EC monolayer				
				1	2	3	4	3 + 4
		min						
A	Sedimentation	5	4.6*	<1 (4.4)‡	<1 (8.7)	4 (86.9)	0 (0)	4 (86.9)
	by gravity	10	250	14 (5.6)	31 (12.4)	184 (73.6)	21 (8.4)	205 (81.9)
	at 4°C	15	306	16 (5.2)	49 (16.1)	163 (53.2)	78 (25.4)	241 (78.7)
B	Centrifugation	5	200	14 (7.1)	32 (16.0)	154 (76.9)	0 (0)	154 (76.9)
	at 4°C	10	308	19 (6.2)	49 (16.0)	213 (69.0)	27 (8.8)	240 (77.8)
		15	398	15 (3.7)	33 (8.3)	214 (53.7)	136 (34.4)	351 (88.1)
C	None (no initial	5	27	2 (7.4)	4 (14.8)	20 (75.1)	<1 (2.7)	21 (77.8)
	contact)	10	86	2 (2.5)	15 (17.9)	42 (49.4)	26 (30.2)	68 (79.6)
		15	137	5 (3.3)	10 (7.0)	42 (30.8)	80 (59.0)	123 (89.8)

Purified monocytes were prepared (Materials and Methods) and suspended in M199 at densities (1.5×10^6/0.5 ml) that provided a final ratio of >10 monocytes per EC. This exceeds the maximal number that can bind (1). In the first two techniques (A and B), both EC monolayers and monocytes were chilled to 4°C. Monocyte-EC binding is inhibited at this temperature (1). The monocytes were then allowed to settle gently by gravity sedimentation (A) or rapidly by centrifugation (B) over 5 min while still at 4°C. Then beginning at time 0, the monolayers were warmed to 37°C to initiate binding. In the final method (C) monocytes were added in suspension (23°C) to EC-collagen monolayers (37°C) and incubated (37°C) immediately. Therefore, in contrast to methods A and B, there was no initial contact at time 0, and the incubation time in C included both time to settle and to attach. There was, however, no initial inhibition of binding nor extreme temperature exposure for the EC in method C. At the indicated times, the monolayers were fixed with gluteraldehyde and stained with silver and Diff-Quick before examination by light microscopy. The results presented are representative of three experiments. The location of bound monocytes was scored as depicted: (*1*) monocytes bound at greater than one monocyte diameter away from an EC margin; (*2*) monocytes bound within one monocyte diameter from the silver stained border but not in contact with it; (*3*) monocytes overlying the silver lines to any degree; (*4*) monocytes penetrating the monolayer. The latter could be appreciated easily by focusing into the monolayer and using the silver line as a marker for the plane of the EC monolayer at that point. Monocyte distribution was not affected by possible rolling down the physical elevation corresponding to EC nuclei since nuclei were found randomly located within individual EC (center versus periphery) and monocytes were often found adherent above or near a nucleus located at the periphery of an EC. Because the collagen gels tended to form a meniscus, monocyte attachment was determined both at centers and the periphery. The results for location were similar when analyzed separately.

* Average number determined from counts of 10 individual fields; where the number of monocytes per field was >100, only 5 individual fields were assessed.

‡ Numbers in parentheses represent percentages of the total number of monocytes counted at that time point.

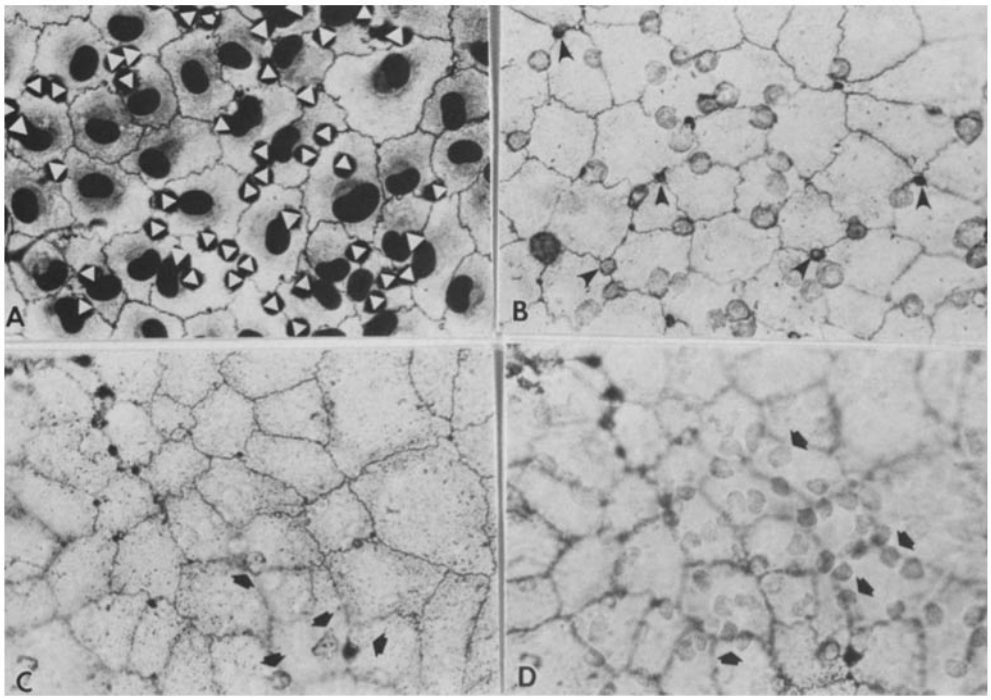

FIGURE 3. Monocyte interaction with EC-collagen monolayers viewed using light microscopy. First passage, confluent HUVEC cultured upon collagen were incubated with purified human monocytes suspended in M199 (five per EC ratio) for increasing periods of time. Monocyte-EC contact was established by gentle centrifugation at 4°C and binding was initiated by warming to 37°C. At each time point, the monolayers were fixed in glutaraldehyde, exposed to AgNO3, then counterstained selectively to reveal both monocytes and EC nuclei (Diff Quick, panel *A*) or monocytes alone (Giemsa, panels *B–D*). (*A*) After a 5-min incubation, monocytes were most likely located at their initial binding sites (discussed in Results). Most of the adherent monocytes (distinguished from EC nuclei by *white arrowheads*) were attached to the margins of EC. (*B*) After a 10-min incubation, most monocytes remained above the plane of the EC monolayer, as determined by focusing above and below the grid formed by the silver lines. However, occasional monocytes were seen passing between EC at this time point (*arrows*) (Table II). (*C* and *D*) After 60 min, few monocytes remained above the grid (*arrows* in *C*). By focusing down into the gel (*D*), most of the monocytes were found in locations immediately below the EC (*arrows*). *A–D*, ×280.

layers was also examined using SEM and TEM (Figs. 4–8). As with TEM (Fig. 2), extensive overlapping of EC was evident under SEM (Fig. 4 *A*). The preferential binding of monocytes at early time points to EC margins rather than "centers" (Table II) was confirmed by both SEM (Fig. 4 *B*) and TEM (Fig. 5 *A*). In separate preparations in which monocytes were incubated with EC-collagen monolayers for 5 min, a total of 50 individual monocytes were identified under TEM. 90% were overlaying intercellular junctions, or had a membrane extension in contact with a junction, thus confirming the results obtained using silver staining and light microscopy (Table II).

Monocyte translocation between EC was also examined under TEM (Fig. 5). After passage through the monolayer was complete, monocytes appeared first to orient

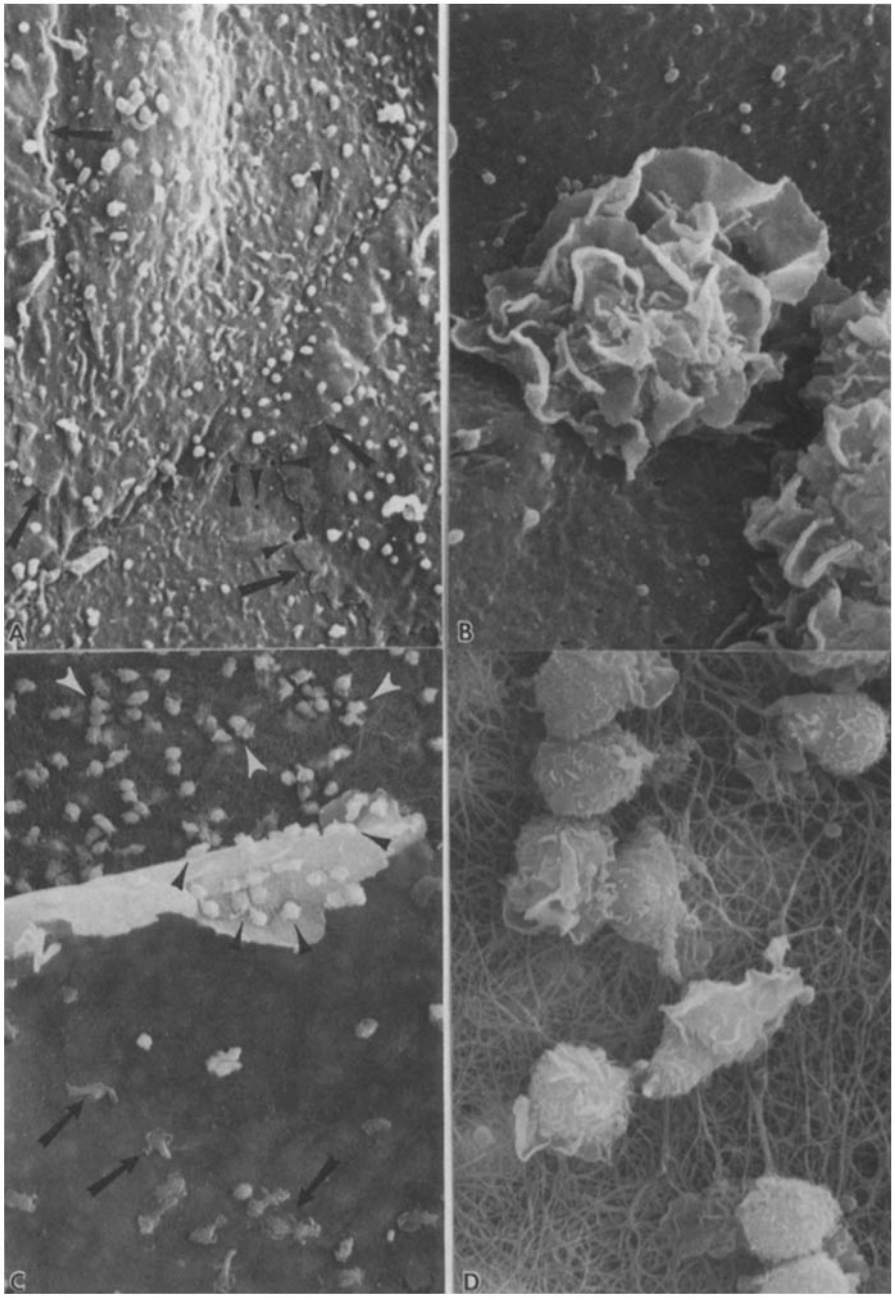

FIGURE 4. Monocyte migration through EC-collagen monolayers viewed under SEM. (*A*) Prominent features of the luminal surfaces of EC included numerous villous projections, a few apertures of forming vesicles (*arrowheads*), and overlapping cell margins (*arrows*). ×4,250. (*B*) Monocytes adherent to EC surfaces are well spread with extensive membrane ruffling. ×4,500. (*C*) In this view (near 90° angle) of an EC monolayer incubated with monocytes for 45 min, an accidental tear during processing provided exposure of monocytes at three distinct phases of their migration. A few spread and oriented monocytes remained on the EC surface (*arrows*). After passage through the monolayer, monocytes remained oriented to the basilar surfaces of EC (*arrowheads*) before they migrated further into the collagen (*white arrowheads*). ×330. (*D*) Under higher magnification, monocytes appeared to traverse the collagen meshwork by extending lamellipodia. ×2,200.

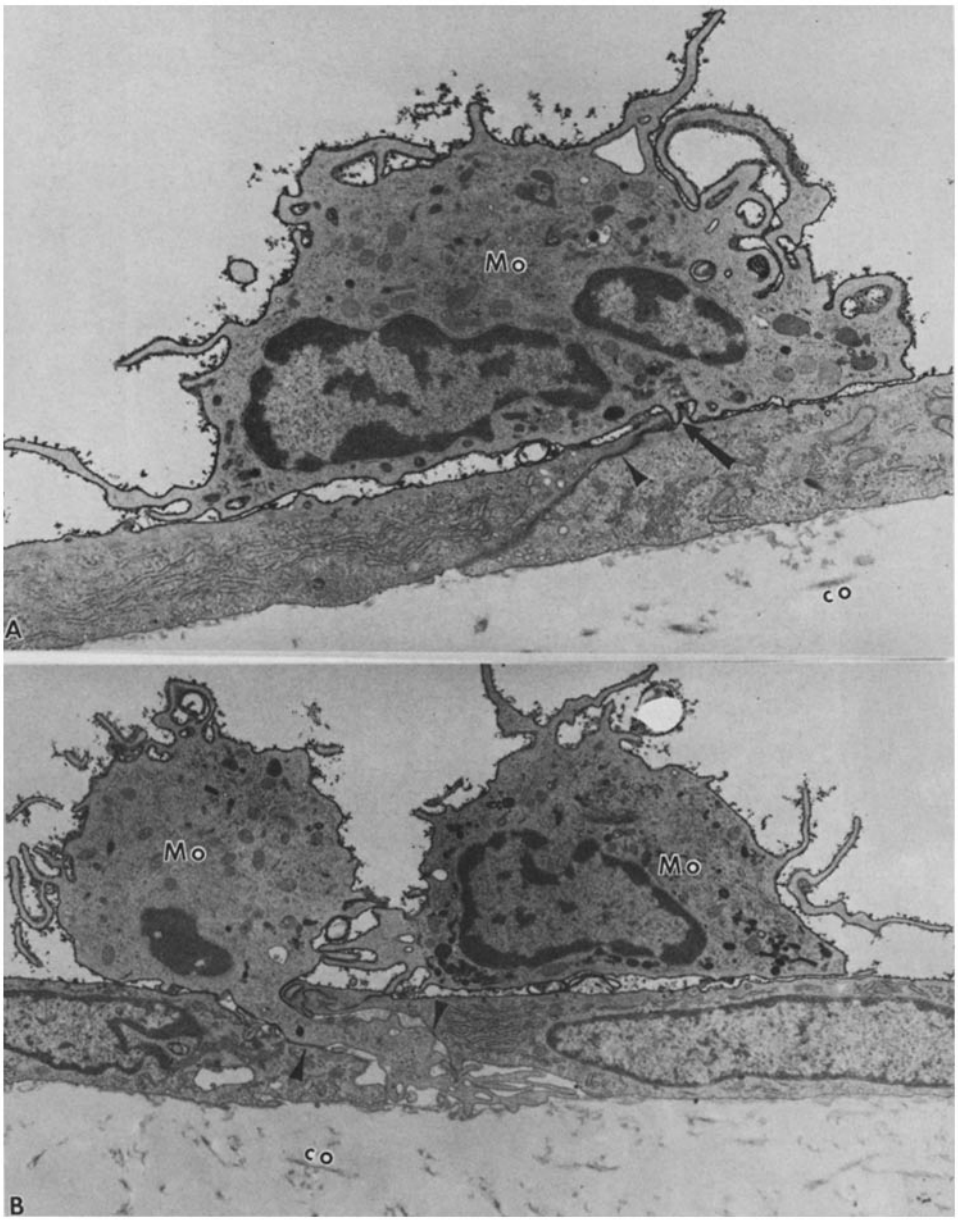

FIGURE 5. Monocyte penetration of EC-collagen monolayers. (*A*) Monocytes (*Mo*) establish extensive membrane contact with the apical surfaces of EC. Penetration appeared to begin by the insertion of a pseudopod between two EC (*arrow*). Note the presence of complex junctional structures between the EC. ×9,000. (*B*) Monocytes (*Mo*) pass between EC by "squeezing" through a narrow channel and maintaining extensive membrane contact (*arrowheads*); co-collagen. ×7,000.

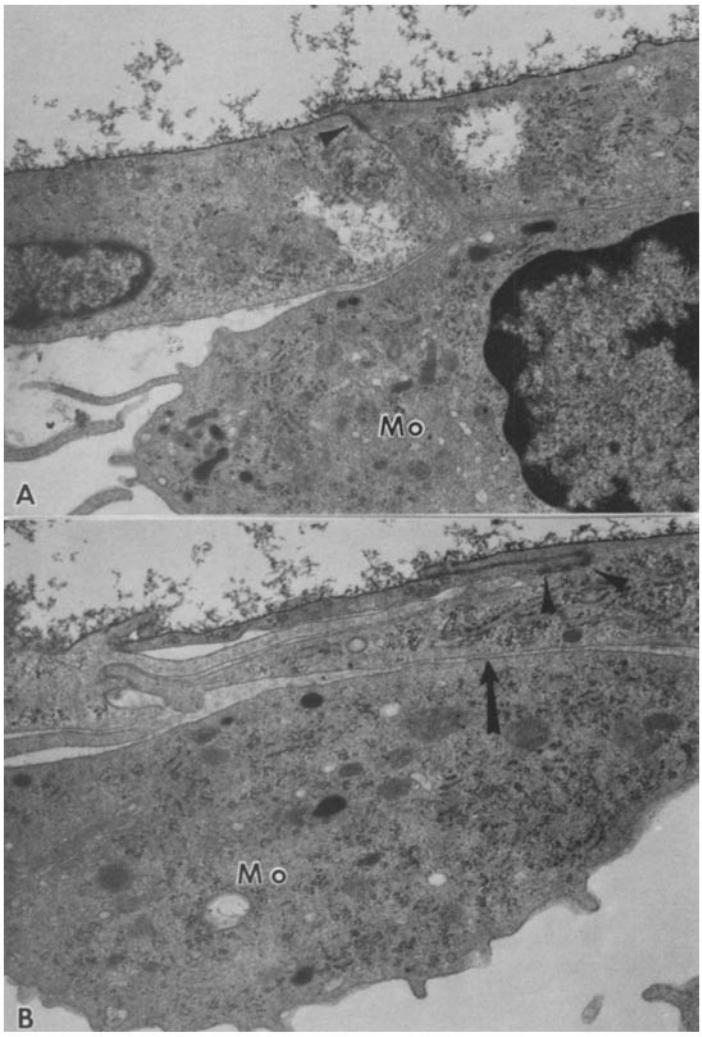

FIGURE 6. Specialized intercellular junctions were found immediately adjacent to spaces through which monocytes had just passed. (*A*) Complex junctional structures can be found in intercellular spaces soon after monocyte (*Mo*) transit is completed (*arrowhead*). (*B*) Note the extensive membrane contact established by monocytes (*Mo*) with the basilar surfaces of the EC (*arrows*). Junctional adhesion plaques have reformed (*arrowheads*). *A*, ×13,000; and *B* ×16,000.

to the basal surfaces of the overlying EC (Figs. 4 *C*, 6, and 7), and at later times they appeared to randomly migrate deeper into the collagen matrix itself (Fig. 4 *D*). During all phases of penetration between EC, monocyte and EC plasma membranes remained in close contact (Fig. 5 *B*). This and the apparent persistence of silver reaction product encircling migrating cells (Fig. 3 *B*) suggest the maintenance of a "tight seal" during this process. Notably, specialized intercellular junctions were seen in sections containing monocytes that appeared to have just completed their passage through the monolayer (Fig. 6, *A* and *B*).

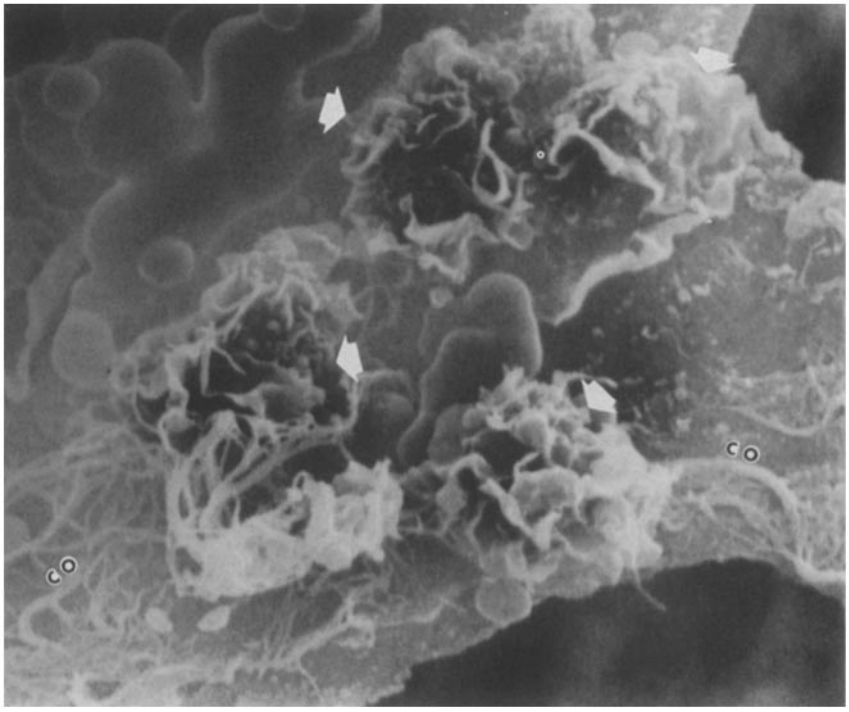

FIGURE 7. Monocyte interaction with the underside of EC-collagen monolayers. Detail from the underside of the monolayer described in Fig. 4. Before migration into the collagen matrix, the monocytes (*arrows*) established extensive membrane contact with the basilar surfaces of EC and appear "spread." co-collagen fibers. ×3,750.

Discussion

Leukocyte emigration from within the vasculature involves a sequence that includes adherence to luminal surfaces of EC, translocation through the endothelium, interaction with basement membrane structures, and finally, migration deeper into the extracellular matrix (ECM). Basal rates may be amplified during inflammatory responses by alterations in the leukocytes themselves and the EC barrier, as well as by increases in circulating cells and alterations in flow. Many questions remain about each of these details, and their investigation would benefit from in vitro models using homologous cell types that exhibit characteristics that reflect their native state in vivo. In previous work we found that monocytes interact with EC with high affinity (1). We have now found that human monocytes retain their high affinity for HUVEC cultured upon a collagen gel matrix. In this model, in contrast to protein-coated plastic surfaces, confluent EC stain with metallic silver and form complex intercellular junctions with high frequency.

Endothelium that has developed in vivo upon a native ECM uniformly stains with silver (15, 17). This staining characteristic therefore has come to indicate a state of maturation consistent with its function as a barrier or specialized epithelium. Highly organized intercellular junctions and the establishment of apical/basilar polarity might result. The basis for the silver-staining reaction itself remains unknown (18). It has

been suggested that silver binds to substances on EC surfaces, intercellular spaces, and the basal lamina. Interestingly, indigocarmine and Azure II (applied sequentially) stain native endothelium (15) as well as EC-collagen monolayers (not shown). Areas in which EC have detached from EC-collagen monolayers also stain intensely with both of these procedures. Monocytes also adhere more vigorously to such denuded areas, which suggests a direct relationship between the deposition of stain and the matrix components for which monocytes have highly affinity. This relationship appears to be more indirect since monocytes bind avidly to silver stain-negative, senescent EC in EC-collagen monolayers as well as EC grown on plastic that do not stain at all. It is more likely that any number of ECM components or other cell surface ligands to which monocytes adhere, such as laminin and elastin (24), co-distribute with the ECM components that bind silver. The capacity of EC to bind silver was, however, dependent on the presence of calcium. Upon its depletion to the micromolar range, silver staining was abolished. Similar results were obtained after a brief exposure to cytochalasin D (not shown). In either case, the capacity to bind silver is regenerated after return to basal medium within several hours. Interestingly, exposure to vasoactive agents such as histamine, thrombin, and leukotriene C did not disrupt silver staining of EC-collagen monolayers.

A positive silver staining characteristic has been noted in several other in vitro models using bovine and human EC (25–28). In many of these, the formation of complex intercellular junctions was also documented. In additional studies (29–31), morphology of junctional areas was explored, but not affinity for silver. However, it is apparent that the silver stain-positive models do not share a common growth substratum (gelatin, amnion, collagen). The one common feature is rather a growth surface that is porous (filter membranes) or at least permeable to a basilar compartment. This is consistent with the fact that silver staining develops on HUVEC grown on gelatin spheres (28) but not on gelatin-coated plastic surfaces. Perhaps such a physical construct better allows for the development of an apical/basilar polarity and its associated vectorial transport and secretory activities. Although a confluent state is not required for the establishment of some surface characteristics indicative of apical/basilar polarization (32), Zerwes and Risall (27) have demonstrated that EC grown on a porous membrane develop silver-stainable, complex intercellular junctions as well as directed secretion of chemotactic material to basilar surfaces.

Vascular beds differ in the complexity and numbers of intercellular junctional complexes they express (33–34). HUVEC develop in situ three types of junctional complexes: adherens junctions with and without spot fusions and classic pentalaminar fusions characteristic of occlusive or tight junctions (21). In one study, limited morphologic assessment of junctional complexes of HUVEC in situ and in vitro were performed. Apparently, similar types junctional specializations were found in primary cultures (35). It is nonetheless clear that junctional areas in HUVEC monolayers grown on plastic are not well developed (36). The HUVEC monolayers grown on collagen in this study express with high frequency the same types of junctional specialization noted for HUVEC in situ.

A number of studies have now addressed human monocyte-EC binding in vitro (1, 2, 23, 26, 37–40). Although monocyte affinity for EC appears to vary between species of EC (2, 37–40), monocytes consistently were found to bind avidly to cultured HUVEC. When provided in excess under static conditions for at least 60 min,

maximal monocyte binding reaches 5–8 per EC, whereas neutrophils remain at the level of 1 per EC (1). This occurs regardless of the growth matrix provided for EC (plastic, fibronectin, gelatin, collagen, or amnion), added growth factors, or the suspending medium present during binding assays (medium alone, serum, or plasma).

The binding of monocytes to the margins of individual EC strongly suggests a specialized organization of the apical surfaces of the EC. In preliminary experiments, agents that amplify monocyte-EC binding (IL-1 and LPS) did not alter this specific pattern of binding to EC margins. Precedent exists for such a unique topography in the peripheral distribution of histamine receptors on the luminal surfaces of EC (41) and the specialized distribution of anionic sites (42). It may be that high affinity binding sites are situated near intercellular junctions, and represent one pole of a chemotactic gradient that directs bound cells to cell junctions and below. Selective binding to the junctional zone would then promote a haptotactic substrate along which monocytes would migrate directionally.

Monocytes were found to migrate beneath the monolayers at high basal rates and similar results were found for neutrophils (not shown). This suggests that a significant chemotactic gradient exists for both cell types. It is likely that the EC themselves secrete chemoattractant(s) and EC have been demonstrated to produce a spectrum of such activities (27, 43–45). These possibly could be concentrated below the EC monolayer, either in the fluid phase or bound to matrix components. It was found that in the absence of EC, monocytes bound poorly to collagen matrices in serum-containing medium. However, treatment of collagen gels with EC-conditioned medium enhanced monocyte binding without significantly enhancing penetration into the monolayer.

The two-dimensional view afforded by examination of the silver-stained monolayers under light microscopy demonstrated that monocyte penetration occurs at the intercellular junctions of EC. Our morphologic assessment suggests no significant disruption of the monolayer during monocyte penetration and is in agreement with other in vitro studies of human monocyte (26) and neutrophil (46) interaction with bovine microvascular EC cultured upon amniotic membrane (25). However, it has been suggested that monocyte migration through endothelial monolayers can enhance permeability for macromolecules (47). This issue has not yet been studied in this system. After passage through the endothelium, the monocytes then establish extensive contact with the basilar surfaces of the EC monolayer (Fig. 7) before their random migration deeper into the gels. Once in this enviroment they are able to survive for many weeks and perhaps gain differentiated functions. Finally, the migrating leukocytes may be modified through contact with EC or exposure to matrix components generated by the endothelium. Alteration in surface characteristics and secretory functions of monocytes are possible (48–49). The described model may shed light on the processes that occur during inflammation as a result of leukocyte-EC interactions.

Summary

Human monocytes show a high affinity for vascular endothelium both in vitro and in vivo. To explore monocyte-endothelial interaction in greater detail, we have developed a new in vitro model for growth of human endothelial cells (EC). Human

umbilical vein EC (HUVEC) cultured upon collagen gels form confluent monolayers of EC that bind silver at their intercellular border similar to cells in situ. Intercellular junctional structures, both adherens and tight junctions, were identified. In contrast, HUVEC grown on plastic surfaces did not stain with silver. The silver-staining characteristic of EC-collagen monolayers was reversible and related to their in vitro maturation and senescence. Silver staining of EC borders provided a grid by which the location of monocyte binding to the luminal surface of individual EC could be assessed. Using this technique, we found that monocytes preferentially bound to the margins of EC, in approximation to the silver-staining junctions. These results suggest that EC determinants recognized by monocytes occur in a unique topographical distribution on the apical face of EC. After binding, monocytes migrated through the EC monolayers at high basal rates. The lack of penetration of collagen gels in the absence of an EC monolayer suggested the generation of EC-specific chemotactic signal(s). Monocytes were observed to pass between EC without evidence of disruption of the monolayer. Silver stain remained present during all phases of migration, and under transmission electron microscopy, junctional complexes were found proximal to monocytes that had just completed their passage through the monolayer. After orientation to the basal surface of the EC monolayer, monocytes migrated randomly into the underlying collagen gel. Monocyte adherence, penetration, migration, and long term survival can be studied under these conditions.

We thank the obstetrical staffs at Lenox Hill Hospital, New York, NY, and Chestnut Hill Hospital, Philadelphia, PA, for their assistance in obtaining umbilical cords; Dr. Eva B. Cramer for helpful discussions; and Judy Adams, Ann Gibbons, Deborah Little, and Natale Tonassini for technical assistance.

Received for publication 3 August 1988.

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