

Mononuclear Phagocytes Egress from an In Vitro Model of the Vascular Wall by Migrating across Endothelium in the Basal to Apical Direction: Role of Intercellular Adhesion Molecule 1 and the CD11/CD18 Integrins

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Summary

Little is known about how mononuclear phagocytes (MP) are cleared from sites of inflammation as inflammatory lesions resolve. In this study, the possibility that MP could be cleared from tissues by migrating across endothelium in the basal to apical direction was investigated. In an in vitro model of a blood vessel wall consisting of human umbilical vein endothelial cells (HUVEC) grown on amniotic tissue, a majority of MP that initially transmigrated into the amnion later exited by migrating back across the endothelium in the basal to apical direction. MP that egressed from these cultures adhered to the apical surface of the endothelium or were found nonadherent in the medium above the endothelium. Egression of MP continued throughout the 4-d period examined, displaying higher than first order kinetics and a $t_{1/2}$ of approximately 24 h. These kinetics were decreased by increasing the volume of medium bathing the cultures, suggesting that a soluble factor(s) regulates the rate of egression. In contrast, the kinetics were accelerated by pretreating the endothelium with IL-1. The initial phase of this increased rate of egression was inhibited by antibodies to intercellular adhesion molecule 1 (ICAM-1) or CD18 by 100 and 71%, respectively. Immunostaining revealed that ICAM-1 was present on the apical and basal surfaces of umbilical vein endothelium in vitro and in situ. These data demonstrate that MP can traverse endothelium in the basal to apical direction, and lend insight into the mechanisms by which this process occurs.

Mononuclear phagocytes (MP)¹ are found ubiquitously in animal tissues. Derived from bone marrow precursors, they first circulate in the blood where they are known as monocytes. When monocytes traverse the endothelial lining of blood vessels to enter tissues, they differentiate into macrophages (1). During inflammation, accumulation of MP at the affected site usually increases greatly above the steady state level (2, 3). Once localized to a site of inflammation, MP phagocytize infectious organisms and damaged tissue debris, act as APCs, and secrete a large number of immunoregulatory molecules (4). In conjunction with the resolution of an inflammatory lesion, the density of MP in the affected tissue declines over a period of several days (3, 5, 6). However, little is known about the mechanisms by which accumulated MP are removed from tissues.

Three means for clearance of MP from tissues have been suggested in different experimental models: (a) exit via lymphatic vessels; (b) death in situ and subsequent engulfment by other macrophages; and (c) egression from tissues by reentrance into the bloodstream. With respect to the first of these mechanisms, it has been clearly demonstrated that MP can exit connective tissue (7) or pulmonary tissue (8, 9) through draining lymphatic vessels, but only 0.1–0.2% of MP that accumulate within the lung use such a route (8). Second, phagocytic engulfment by MP is thought to be a major mode of clearance for apoptotic neutrophils (10), but phagocytosis of one MP by another has yet to be described in vivo. Apoptosis of MP in situ has been observed in atherosclerosis (11, 12). In contrast, some monocyte-derived foam cells seem to be cleared from atherosclerotic lesions by migrating across endothelium in the abluminal to luminal direction and consequently reentering the bloodstream (13–15). Whether MP other than lipid-laden foam cells are actually capable of traversing endothelium in the basal to apical direction has not been determined. Thus, the relevance of such a mechanism under steady state or other inflammatory conditions is unknown.

¹Abbreviations used in this paper: HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; IL-1ra, IL-1 receptor antagonist; M199, Medium 199; MCP-1, monocyte chemoattractant protein 1; MP, mononuclear phagocytes; RT, room temperature; VCAM-1, vascular cell adhesion molecule 1.

Although the studies just mentioned suggest otherwise, it has become generally accepted that, once extravasated, MP cannot recirculate (16). However, we are not aware of any data that confirm this premise. In the present study, we explored whether human MP migrate across endothelial monolayers in the basal to apical direction *in vitro*. For these investigations, a well-characterized model of a blood vessel wall that consists of human umbilical vein endothelial cells (HUVEC) grown on human amniotic connective tissue was employed (17). In these experiments, MP did indeed traverse endothelium in the basal to apical direction. An analysis of how this phenomenon was regulated indicated that egression of MP from HUVEC/amnion cultures is not a completely random process, since its kinetics could be altered by cytokines or by dilution. Further studies are presented that indicate that MP use CD11/CD18 to bind to endothelial intercellular adhesion molecule 1 (ICAM-1) as they migrate across the endothelium in the basal to apical direction.

Materials and Methods

Isolation and Culture of Cells. HUVEC/amnion cultures were prepared as previously described (17). In brief, amniotic membranes from human placentas (obtained from normal deliveries at University Hospital, State University of New York [SUNY] at Stony Brook) were fastened to Teflon rings (SUNY at Stony Brook Machine Shop) and held in place by a Viton O-ring (C. E. Conover & Co., Inc., Fairfield, NJ), forming a well-like culture chamber. Individual chambers were soaked in 1.7% NH₄OH (vol/vol), and the bottom surface was scraped with a rubber policeman to remove the amniotic epithelium. The resultant acellular amniotic connective tissue consists predominantly of collagen types I and III (18). The thickness of amniotic tissues used averaged ~90 μ m, ranging from about 40 to 130 μ m. Endothelial cells isolated from human umbilical veins (collected at St. Charles Hospital, Port Jefferson, NY) (19) or human microvascular endothelial cells given by Dr. Marcia G. Tonnesen (SUNY at Stony Brook) were seeded on the stromal surface of prepared amniotic tissue at second or sixth passage, respectively. Endothelial cell-amnion cultures were used in transmigration assays 7–10 d later. HUVEC/amnion cultures were maintained in Medium 199 (M199) (GIBCO BRL, Gaithersburg, MD) containing 20% fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, UT), 100 U/ml of penicillin, 100 μ g/ml of streptomycin, and 2 μ g/ml of amphotericin B (GIBCO BRL). Only serum having undetectable levels of endotoxin, as reported by the manufacturer, was used. We tested the serum further to ensure that it did not activate transendothelial migration of neutrophils (see below and Results).

Human monocytes were isolated to greater than 90% purity by density gradient centrifugation in a hyperosmotic medium (Accudenz; Accurate Chemical Co., Westbury, NY) according to the method of Meerschaert and Furie (20), and neutrophils were isolated by centrifugation of leukocyte-rich plasma in Ficoll-Hypaque as described by Anderson et al. (21).

Transmigration Assay. Isolated monocytes were resuspended in M199 containing 20% heat-inactivated FBS, and transmigration assays were initiated by application of 2×10^5 monocytes/cm² to the apical side of HUVEC/amnion cultures that were either pretreated with 5 U/ml of IL-1 β (Collaborative Biomedical Products, Bedford, MA) for 4 h or left untreated. Cultures treated

with IL-1 were rinsed twice in M199 before addition of monocytes. Three to five cultures were used for each experimental parameter. HUVEC/amnion cultures incubated for more than 2 h with MP were rinsed after 2 h of incubation by sequential passage through three beakers containing M199 (100 ml each). Medium with or without Abs was then added to the interior of the cultures (apical compartment) and beneath the cultures for further incubation. For experiments using Abs, rinsed HUVEC/amnion cultures containing transmigrated MP (2-h cultures) were positioned on silicone rubber supports glued to the bottom of 12-well culture dishes (22) before the addition of medium to the basal compartment. This step to elevate the cultures was included to maximize the access of antibodies to the basal aspect of the endothelium.

After incubation at 37°C for the desired duration, the cultures were fixed in 10% formalin. Fixed cultures were rinsed in saline, cut from the Teflon ring with a cork-borer, and stained with modified Wright's stain. To quantitate the percentage of MP added that became associated with the cultures, stained cultures were viewed en face microscopically and the number of MP in nine $\times 400$ fields per tissue was counted. Cross sections (2.2 μ m) of each culture, embedded in block molds of JB-4 polymer (Polysciences, Inc., Warrington, PA), were examined to determine the percentage of associated monocytes that had migrated beneath the HUVEC monolayer. These values were corrected to account for detachment during the embedding process of MP that were adherent to the apical surface of the endothelium, as previously described (23).

For transmigration studies using neutrophils, neutrophils were incubated with HUVEC/amnion cultures (5×10^5 neutrophils/cm²) for 20 min at 37°C. HUVEC were pretreated with medium conditioned by MP/HUVEC cocultures or freshly prepared medium for 4 h and washed in M199 before application of neutrophils. In other experiments measuring transmigration of neutrophils, unstimulated HUVEC/amnion cultures were elevated on silicone rubber supports glued to the bottom of wells in a 12-well plate, and 10^{-7} M FMLP (Peninsula Laboratories, Inc., Belmont, CA) was added to the basal compartment (24). Quantitation of transmigration was determined as for MP.

Abs and Recombinant Proteins. The hybridoma producing TS1/18 mAb against CD18 (IgG1) (24) was purchased from the American Type Culture Collection (Rockville, MD). Hybridoma cells were injected into the peritoneum of pristane-primed BALB/c retired breeder mice. IgG in the ascitic fluid was purified according to the method of Harlow and Lane (25) except that protein G-Sepharose (Pierce Chemical Co., Rockford, IL) was substituted for protein A-Sepharose to maximize absorption of mouse IgG1 (26). IgG from ascites containing mAb MOPC31c (Sigma Chemical Co., St. Louis, MO), an IgG1 of unknown specificity, was purified by the same protocol. Fab fragments of these and other Abs used were prepared by incubating purified, intact IgG with Sepharose-immobilized papain (Pierce). The digested material was applied to a protein A-Sepharose (Pierce) column to remove Fc fragments and nondigested Ig. The quality of purification and efficiency of Ig fragmentation were analyzed by SDS-PAGE. Purified Fab fragments were quantitated using the bicinchoninic acid protein assay (Pierce). Fab fragments of TS1/18 and MOPC31c were used in transmigration assays at 40 μ g/ml.

A neutralizing mAb against ICAM-1, R.6.5 (IgG2a) (27), was generously provided by Drs. C. Wayne Smith (Baylor College of Medicine, Houston, TX) and Robert Rothlein (Boehringer Ingelheim Corp., Ridgefield, CT). Fab fragments of R.6.5 were

prepared and used in transmigration experiments at 40 $\mu\text{g}/\text{ml}$. For immunostaining, intact R6.5 mAb was used at 20 $\mu\text{g}/\text{ml}$. As an immunostaining control, UPC10 (IgG2a), an anti- β -2-6-linked fructosan Ab, was purchased from Sigma Chemical Co. and used at 20 $\mu\text{g}/\text{ml}$. F(ab')₂ fragments of H18/7, a neutralizing mAb against E-selectin (28), were kindly given to us by Dr. Francis W. Lusinkas (Brigham and Women's Hospital, Boston, MA) and were used in transmigration experiments at 20 $\mu\text{g}/\text{ml}$. Immunostaining for E-selectin was done using a 1:200 dilution of rabbit polyclonal antiserum against human recombinant soluble E-selectin. Distribution of vascular cell adhesion molecule 1 (VCAM-1) on endothelium was investigated using an immunopurified polyclonal Ab against soluble, recombinant VCAM-1 at 20 $\mu\text{g}/\text{ml}$. For transmigration assays, Fab fragments of the neutralizing mAb against VCAM-1 4B9 were used at 20 $\mu\text{g}/\text{ml}$ (29). These Abs to E-selectin and VCAM-1 were the generous gift of Dr. Roy Lobb (Biogen Inc., Cambridge, MA). For immunostaining controls, normal rabbit serum, obtained from Sigma Chemical Co., was used at equivalent IgG concentrations as these Abs. S101, a neutralizing mAb to monocyte chemoattractant protein 1 (MCP-1) (23), was purchased from Anogen (Mississauga, ON, Canada), digested to Fab fragments, and used in experiments at 20 $\mu\text{g}/\text{ml}$. Ab fragments of mAb TS1/18, MOPC31c, R6.5, H18/7, 4B9, and S101 were tested for endotoxin using a chromogenic limulus amoebocyte lysate assay (BioWhittaker, Inc., Walkersville, MD). When specified, Fab fragments prepared from neutralizing antibodies to TNF- α (R&D Systems, Inc., Minneapolis, MN) were added to HUVEC/amnion cultures at 20 $\mu\text{g}/\text{ml}$, in combination with 100 ng/ml of recombinant human IL-1 receptor antagonist (IL-1ra) (R&D Systems, Inc.). Recombinant human MCP-1 and a sandwich ELISA kit to detect human IL-1 β were also purchased from R&D Systems, Inc.

Immunostaining. To immunostain for adhesion molecules in HUVEC/amnion cultures, cultures were fixed in freshly prepared, neutralized, phosphate-buffered 3% paraformaldehyde (Sigma Chemical Co.) for 1 h at 4°C and then rinsed in PBS. Before application of Abs, cultures were incubated with PBS containing 0.2% BSA (Sigma Chemical Co.) for 30 min at room temperature (RT). Then dilutions of the antiadhesion molecule Abs were applied to the cultures for 2 h at RT. Subsequently, cultures were rinsed in PBS and then washed for 1–12 h in 500 ml of gently stirred PBS at 4°C. TRITC-conjugated goat antimouse or anti-rabbit IgG (Organon Teknica Corp., Durham, NC) was added to the cultures at 8 $\mu\text{g}/\text{ml}$ for 1 h at RT. Finally, the cultures were washed in PBS as described in the preceding step. The tissues were cut from the Teflon ring with a cork-borer and embedded in JB-4 polymer for cross sectional examination.

To immunostain for adhesion molecules in intact umbilical veins, the central vein of umbilical cords was cannulated, perfused with M199 containing 20% heat-inactivated FBS with or without 5 U IL-1/ml, and incubated at 37°C for 4 h (for E-selectin immunostaining) or 18 h (for ICAM-1 and VCAM-1 immunostaining). Umbilical veins were then perfused with PBS containing 3% paraformaldehyde and incubated for 30 min at RT to fix the vein in a distended position. Finally, the entire umbilical cord was immersed in the fixative solution for an additional 30 min. The tissue was rinsed in PBS and cut into thin slices (\sim 3 mm) with a razor blade. Immunostaining was then carried out as just described. This procedure was performed using umbilical cords obtained within 2 h of delivery.

Chemotaxis Assay. Solutions of conditioned medium from MP/HUVEC cocultures, containing or lacking Fab fragments to MCP-1, were placed in the lower compartment of blind well

chambers (Neuroprobe, Inc., Cabin John, MD). Freshly isolated monocytes were resuspended in M199 containing 20% heat-inactivated FBS and were placed in the upper compartment of the chambers, which was separated from the lower compartment by a cellulose nitrate filter with 5.0- μm pores (Neuroprobe, Inc.). The chambers were incubated for 90 min at 37°C, and filters were fixed, stained, and analyzed as previously described (23).

Statistics. The percentages of MP beneath the endothelium in experimental conditions and controls were compared using the two-tailed Student's *t* test. Data from different experiments were normalized before comparison by setting the highest value for percentage of MP located beneath the HUVEC monolayer within each experiment equal to 1.0 and calculating relative values for the remaining data.

Results

MP Egress from a Tissue Substrate by Migrating across Endothelium in the Basal to Apical Direction. Previous work from this laboratory has demonstrated that isolated human monocytes traverse unstimulated HUVEC monolayers grown on amniotic tissue (20). Maximal transmigration of monocytes is reached after 2 h, when \sim 40% of monocytes added to HUVEC/amnion cultures have crossed the endothelium and are located within the amniotic tissue. Most of the remaining 60% of monocytes do not attach to the endothelium at all. That is, $>$ 95% of monocytes that bind to the apical surface subsequently migrate into the underlying amnion. After traversing the endothelium, MP migrate to a variety of depths within the amnion, but none migrates deeper than about two thirds of the amnion's thickness. Because monocytes likely begin differentiation into macrophages immediately after transendothelial migration, we refer to transmigrated monocytes as MP.

To evaluate whether MP that had entered the amniotic tissue might later exit by migrating back across the endothelium, we performed extended time courses in which MP were incubated with HUVEC/amnion cultures for more than 2 h. In these experiments, monocytes were first incubated with HUVEC/amnion cultures for 2 h. Cultures were then rinsed to remove nonadherent monocytes, and fresh medium was added for further incubation. Relative to 2 h of incubation, there were fewer MP associated with HUVEC/amnion cultures after 12 or 24 h of incubation (Fig. 1). Nonadherent MP could be collected from the apical compartment of the cultures at these later times. Furthermore, a large fraction of MP that remained associated with HUVEC/amnion cultures at 12 or 24 h were found adherent to the apical surface of the endothelium. Indeed, only 66 or 49% of MP that were beneath the endothelium at 2 h of incubation remained there after 12 or 24 h of incubation, respectively (Fig. 1). These data demonstrate that monocytes exited from amniotic connective tissue by migrating across endothelium in the basal to apical direction. Once egressed, MP remained adherent to the apical surface of the endothelium for some period of time and then finally detached (data not shown). It is interesting to note that MP within the amnion were found at a variety of

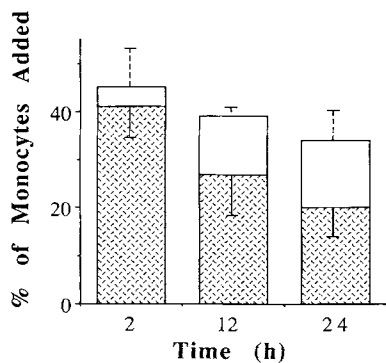


Figure 1. Extended incubation of MP with HUVEC/amnion cultures. MP were incubated with unstimulated HUVEC/amnion cultures for 2 h. After this time, some of the cultures were fixed for analysis of transmigration; other cultures were rinsed thoroughly to remove nonadherent MP, and fresh medium was added for an additional 10 or 22 h of incubation. Total height of the bars represents the percentage of MP added that were associated with the cultures after fixation. Patterned portions of the bars correspond to the percentages of added MP that migrated beneath the endothelium, and open portions of the bars indicate the percentage of MP found adherent to the endothelial apical surface. Plotted values represent the mean \pm SD of triplicate cultures from 2 (for 12 h data) to 10 (for 24 h data) independent experiments. SDs for values indicating the percentage of MP associated with HUVEC/amnion cultures are shown by dotted lines (positive direction only). SDs for values indicating the percentage of MP located beneath the HUVEC monolayer are depicted by solid lines (negative direction only). Differences in the numbers of MP beneath the endothelium at 2 h versus 12 or 24 h of incubation are statistically significant, $p < 0.001$. The differences in the total numbers of MP associated with the cultures (total height of the bar) at 2 h versus 12 or 24 h are not significant.

depths, even as some were leaving the cultures. Similar results as shown in Fig. 1 were obtained when MP were coincubated with human microvascular endothelial cells grown on amnion (not shown).

The kinetics of egression of MP from HUVEC/amnion

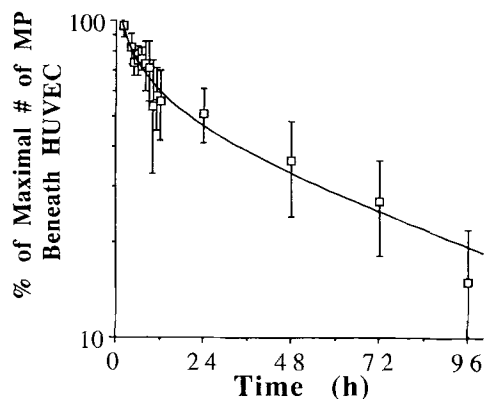


Figure 2. Time course of MP retention in amniotic connective tissue. HUVEC/amnion cultures were incubated with MP for 2 h, rinsed, and fixed or further incubated for an additional 2–94 h. Cultures incubated longer than a total of 24 h were rinsed thoroughly at each 24 h interval to remove nonadherent MP from the apical compartment of the cultures. Data were normalized by setting the values obtained at 2 h of incubation (when there was a maximal number of MP beneath HUVEC) in each experiment equal to 100% and calculating relative values for the remaining data. Data are combined from five experiments performed in triplicate. r for logarithmic fit of curve = 0.98.

cultures were next studied in detail. Egression continued throughout the 4-d period examined, albeit relatively slowly after the first 24 h, displaying higher than first order kinetics and a $t_{1/2}$ of ~ 24 h (Fig. 2). Analysis of some of the cultures by trypan blue exclusion revealed that $>95\%$ of MP and HUVEC were viable during the 4-d assay. MP that remained within amniotic tissue for at least 48–72 h underwent pronounced morphological changes, acquiring a fusiform shape and increasing in size (Fig. 3). These morphological changes most likely resulted from their ongoing

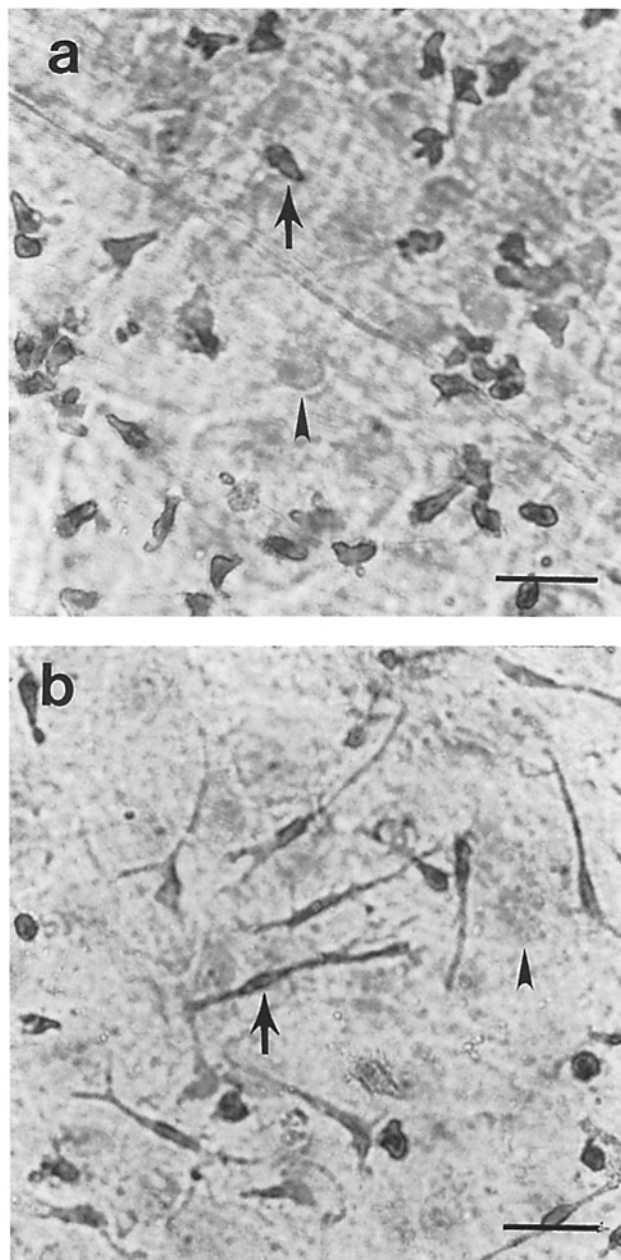


Figure 3. Micrographs of MP/HUVEC cocultures viewed en face. (a) The morphology of MP incubated for 2 h with HUVEC grown on human amniotic connective tissue; the MP have just completed their transmigration beneath the endothelium. (b) The typical morphology of a MP-HUVEC coculture after 72 h of incubation. (Arrows) Representative MP. (Arrowheads) Faintly apparent endothelial cell nuclei. Bar, 30 μm .

differentiation into macrophages. Because egression progressed beyond the time when these morphological changes occurred, these data suggest that differentiation into macrophages may not abrogate the potential of MP to exit connective tissue by migrating across endothelium in the basal to apical direction.

IL-1 Increases the Initial Rate of Egression of MP from HUVEC/Amnion Cultures. IL-1 β is a proinflammatory cytokine that acts on endothelial cells to upregulate adhesion molecules and chemoattractants for leukocytes (30). We, therefore, investigated the effect of IL-1 on egression of MP from HUVEC/amnion cultures. Previous studies have shown that IL-1 does not increase the extent of monocytic transmigration across HUVEC monolayers in the apical to basal direction, though it does accelerate the kinetics of their adhesion (20). Pretreatment of HUVEC with IL-1 increased the rate at which monocytes egressed from the cultures (Fig. 4). The increased kinetics caused by IL-1 were apparent only in the first few hours of the assay; after 4 h of incubation, the rate of exodus was similar in IL-1-treated and untreated cultures.

Because MP are primary sources of proinflammatory cytokines such as IL-1 *in vivo* (31), it was important to determine whether MP that migrated across unstimulated HUVEC/amnion cultures produced IL-1 or other cytokines with similar proinflammatory activity (such as TNF- α), which in turn might act on the endothelium to promote egression of MP from the cultures. To detect the biologic activity of proinflammatory cytokines, an assay measuring transmigration of PMN was used. PMN incubated with HUVEC/amnion cultures migrate across endothelial monolayers pretreated with IL-1 or TNF- α but not across unstimulated HUVEC (32). In this assay, PMN were stimulated to transmigrate across HUVEC pretreated with

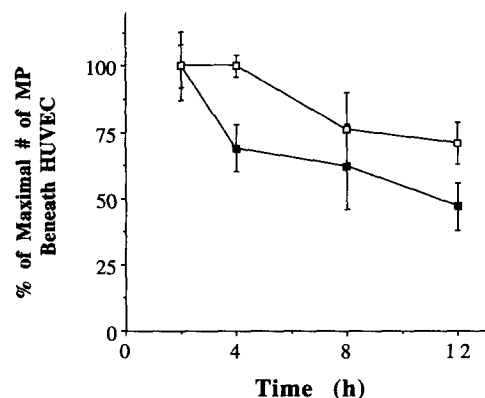


Figure 4. Effect of IL-1 on exit of MP from HUVEC/amnion cultures. MP were incubated for 2 h with untreated HUVEC/amnion cultures (open symbols) or HUVEC/amnion cultures that had been pretreated with IL-1 for 4 h (filled symbols). Cultures were then fixed or rinsed thoroughly in M199 and further incubated for 2–10 h. Data plotted are derived from a single experiment performed in triplicate. Data were normalized as in Fig. 2. The percentage of monocytes that transmigrated into unstimulated or IL-1-stimulated HUVEC/amnion cultures at 2 h of incubation was $51 \pm 4\%$ and $45 \pm 6\%$, respectively. The comparison at 4 h of incubation in this experiment was repeated five times with similar results.

conditioned medium from MP/HUVEC cocultures (Fig. 5), indicating that proinflammatory cytokines are indeed produced by MP/HUVEC cocultures. A sandwich ELISA confirmed the presence of IL-1 in the cultures at levels that ranged from 7 to 96 pg/ml in the conditioned media collected.

The production of proinflammatory cytokines by cocultures of MP and HUVEC raised the question of whether egression could occur in the absence of cytokine stimulation of the endothelium. Perhaps basal to apical transendothelial migration of MP is a cytokine-dependent process and occurs at a later time in the initially unstimulated cultures, relative to cultures pretreated with IL-1, because of a delay in the synthesis of IL-1 or TNF- α *in situ* by the MP. To assess the requirement for stimulation by cytokines, we incubated MP/HUVEC cocultures for 13 h in the presence of neutralizing anti-TNF- α Fab fragments and IL-1ra, which prevents binding of IL-1 to IL-1 receptors (31). The extent of exit of MP from HUVEC/amnion cultures in the presence of these inhibitors was not different than from cultures lacking the inhibitors (Fig. 5). To be certain that endothelial activation was inhibited in these experiments, subsequent control experiments measuring transmigration of PMN were performed (Fig. 5). In these experiments, fresh HUVEC/amnion cultures were pretreated for 4 h with medium collected from MP/HUVEC cocultures that had been assayed for egression in the presence or absence of IL-1ra and anti-TNF- α Fab fragments. The levels of IL-1ra and anti-TNF- α Ab fragments that remained in medium collected at the end of the egression experiments substantially reduced activation of the endothelium. Specifically, transmigration of neutrophils was 82% lower in HUVEC

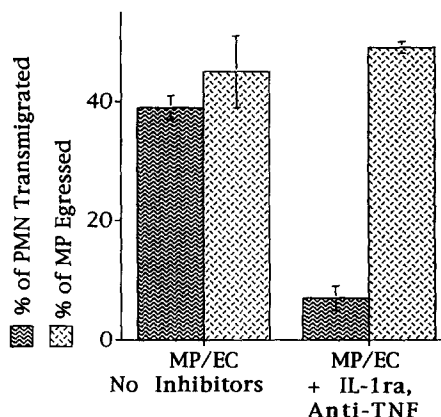


Figure 5. Role of endogenous IL-1 and TNF- α in egression of MP from HUVEC/amnion cultures. MP were added to initially unstimulated HUVEC/amnion cultures in the presence or absence of IL-1ra and neutralizing anti-TNF- α Fab fragments and incubated for 13 h. Cultures fixed at this time were analyzed to determine the extent of MP exodus in the presence or absence of the cytokine inhibitors (stippled bars). Media collected from the MP/HUVEC cocultures at the end of the 13-h assay or fresh medium were incubated with unstimulated HUVEC/amnion cultures for 4 h, and PMN were subsequently added to the cultures to measure their transmigration (striped bars). In HUVEC/amnion cultures preincubated with fresh medium, $0.5 \pm 0.5\%$ of PMN transmigrated. Data represent the mean \pm SD of one experiment performed in triplicate. The experiment was repeated with similar results.

cultures pretreated with MP/HUVEC conditioned medium containing the cytokine inhibitors than in cultures pretreated with medium collected from MP/HUVEC cocultures lacking cytokine inhibitors (Fig. 5). Since emergence of MP from HUVEC/amnion cultures was not suppressed in the continuous presence of inhibitors of endothelial activation, basal to apical transendothelial migration does not seem to require cytokine stimulation of the endothelium. However, we cannot eliminate the possibility that low, residual levels of proinflammatory cytokine activity mediate exodus of MP from HUVEC/amnion cultures.

Experiments using IL-1 α and anti-TNF- α Fab fragments alone or in combination revealed that the majority of proinflammatory cytokine activity that accumulated in MP/HUVEC cocultures was IL-1 β (data not shown). Analyses of media collected from MP cultured for 24 h in amnion alone or under nonadherent conditions in a Teflon beaker delineated the step at which MP were stimulated to produce IL-1. A sandwich ELISA did not detect IL-1 β in 24-h conditioned medium derived from MP adherent to tissue culture plastic or cultured under nonadherent conditions, but such activity was present in medium from MP cultured with amnion lacking endothelium (data not shown). Because only some of these conditions resulted in production of proinflammatory cytokines, secretion of IL-1 by MP is apparently not a direct consequence of the procedure used for their isolation. Collagen, in the presence of fibronectin, stimulates secretion of IL-1 by MP in vitro (33), potentially explaining these findings.

Dilution Decreases the Rate of MP Exit from HUVEC/Amnion Cultures. If egression of MP has physiological relevance, the phenomenon should persist under nonstatic, diluting conditions, as would be found in flowing blood. To investigate this possibility, MP were first allowed to migrate for 2 h across HUVEC grown on amnion. Then the cultures were pierced with a stainless steel hook that was subsequently suspended in a beaker containing 0.5–1.0 liters of culture medium and incubated for 22 or 46 h more with gentle stirring. Cultures to be incubated for a total of 48 h were transferred to a beaker containing 0.5–1.0 liters of fresh medium at 24 h of incubation. These particulars are hereafter denoted as “large volume” conditions. For comparison, MP were incubated with HUVEC/amnion cultures held under the static, smaller volume conditions used in previous experiments (0.5 ml of medium in the interior of the cultures, 1.0 ml of medium surrounding the exterior of the cultures). Such cultures are henceforth stated to have been incubated under “small volume” conditions. The number of MP located within the amniotic matrix of cultures subjected to large volume conditions diminished with time of incubation, but this decrease occurred with slower kinetics than in the small volume controls (Fig. 6), increasing the $t_{1/2}$ of egression to \sim 48 h. By 48 h, the extent of loss in small and large volumes was similar. As in unstimulated cultures, the kinetics of egression in HUVEC/amnion cultures pretreated with IL-1 were markedly reduced under large volume conditions (Fig. 6). However, as in small vol-

ume cultures, the rate of egression of MP from IL-treated cultures incubated under large volume conditions was more rapid in the first few hours than at later times (Fig. 6).

To examine whether the reduced kinetics of egression in large volume cultures resulted from a shear stress response induced by the nonstatic environment or whether the dilution itself could account for the slower exit of monocytes, these experiments were repeated as described except that the medium bathing the suspended cultures was not stirred. The results obtained from this approach were identical to the experiments in which stirring was included (data not shown). Therefore, the decreased rate of basal to apical migration of monocytes across endothelium in large volume cultures appears to result from dilution of a soluble mediator(s).

Role of Adhesion Molecules in Egression of MP from HUVEC/Amnion Cultures. To explore the mechanism by which MP traverse endothelium in the basal to apical direction, the role of adhesion molecules was investigated. First, we

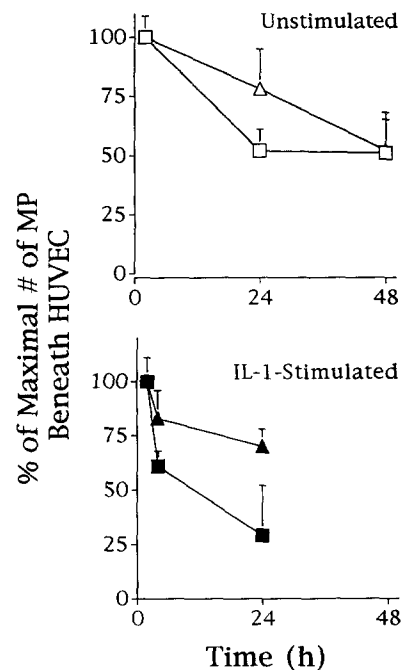


Figure 6. Effect of dilution on the kinetics of MP egression from HUVEC/amnion cultures. Monocytes were incubated with untreated (open symbols) or IL-1-stimulated (filled symbols) HUVEC/amnion cultures for 2 h. Some cultures (Δ , \blacktriangle) were then suspended from stainless steel hooks in a beaker containing 0.5–1.0 liter (large volume) of stirring medium and incubated for an additional 22 or 46 h. For comparison, other MP-HUVEC cocultures (\square , \blacksquare) were incubated for an additional 22 or 46 h under the conditions used in previous experiments (small volume), in which 0.5 ml of fresh medium was placed in the interior and 1.0 ml around the exterior of the cultures. Data points represent the means \pm SD from three experiments performed in triplicate. Compared to the number of MP beneath the endothelium at 2 h of incubation, the number of MP located within the amniotic tissue in 24- and 48-h large or small volume cultures was significantly reduced, $p < 0.001$ for each of the conditions plotted. The differences between the extent of MP egression from 48-h large versus small volume cultures, or between 24- versus 48-h small volume cultures, are not significant.

determined whether any endothelial adhesion molecules known to promote leukocytic migration across endothelium in the apical to basal direction were located on the basal surface of the endothelium, where they might mediate basal to apical transmigration of MP. It has been reported previously that ICAM-1 is expressed on both the apical and basal surfaces of HUVEC in vitro, but expression of VCAM-1 is limited to the apical surface (34). We wished to confirm these results in our model system as well as examine the distribution of the cytokine-inducible adhesion molecule, E-selectin (28). These molecules were microscopically visualized in cross sections of IL-1-stimulated HUVEC/amnion cultures by indirect immunofluorescence. E-selectin was present on both the apical and basal endothelial cell surfaces (Fig. 7 *a*). As in the previous study of Oppenheimer-Marks et al. (34), staining for ICAM-1 was also observed on all HUVEC surfaces (Fig. 7 *b*), but VCAM-1 staining was most intense on the apical surface (Fig. 7 *c*). Thus, the locations of ICAM-1 and E-selectin, but not VCAM-1, were consistent with the possibility that they might mediate basal to apical transmigration of MP. Immunostaining for these molecules was determined to be specific because similar dilutions of isotype-matched Abs

(for mAbs) or species-matched sera (for polyclonal antisera) did not stain HUVEC. In addition, positive staining for each of the molecules was absent (for E-selectin and VCAM-1) or decreased (for ICAM-1) in HUVEC/amnion cultures not pretreated with IL-1 (data not shown). The distribution of these adhesion molecules on umbilical vein endothelium in situ matched the observations made in vitro (Fig. 8 and data not shown), negating the possibility that the results obtained in vitro are due to a loss in the capacity of HUVEC to maintain apical and basal polarity through passage in culture.

To test whether these adhesion molecules mediated egression of MP from HUVEC/amnion cultures, Fab or F(ab')₂ fragments of neutralizing mAb against ICAM-1, E-selectin, or VCAM-1 were added to HUVEC/amnion cultures after MP had completed their migration into the amniotic matrix. Immunostaining experiments using anti-E-selectin antisera verified that Abs applied to the cultures as described in Materials and Methods readily gained access to the basal surface of the endothelium. Fab fragments of mAb against ICAM-1 (R6.5) completely blocked egression of MP from IL-1-treated HUVEC/amnion cultures that were incubated with MP for a total of 5 h (Fig. 9 *a*). mAb fragments against VCAM-1 (4B9), E-selectin (H18/7), or an irrelevant antigen (MOPC31c) had no effect (Fig. 9 *a*). When incubation of MP-HUVEC cocultures (IL-1-pretreated HUVEC) was extended to 12 h, anti-ICAM-1 Fab fragments inhibited basal to apical migration of monocytes across endothelium by only 53% (Fig. 9 *b*).

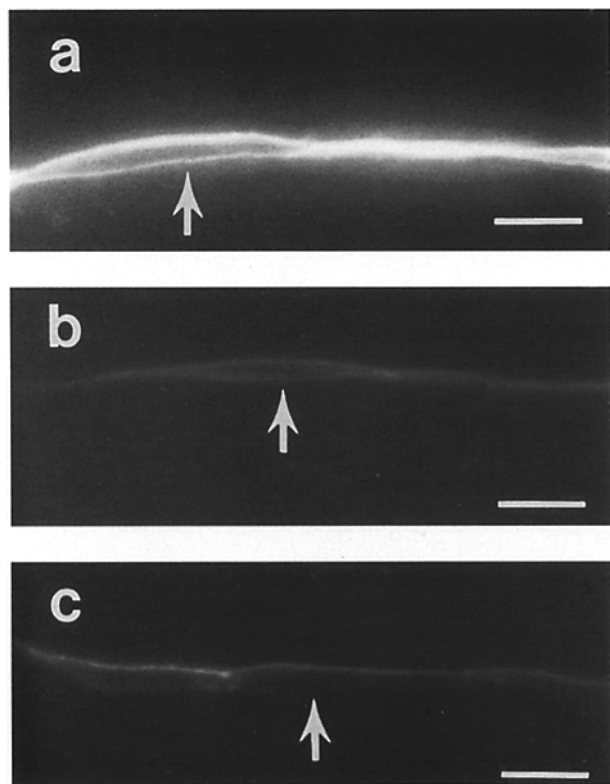


Figure 7. Distribution of adhesion molecules on the apical and basal surfaces of HUVEC in vitro. Abs against E-selectin (*a*), ICAM-1 (*b*), or VCAM-1 (*c*) were incubated with IL-1-stimulated HUVEC/amnion cultures. Subsequent incubation with TRITC-conjugated goat anti-mouse or anti-rabbit IgG permitted visualization of the locations of these adhesion molecules in cross sections of HUVEC/amnion cultures. (Arrows) The basal surfaces of the HUVEC. Bar, 10 μ m.

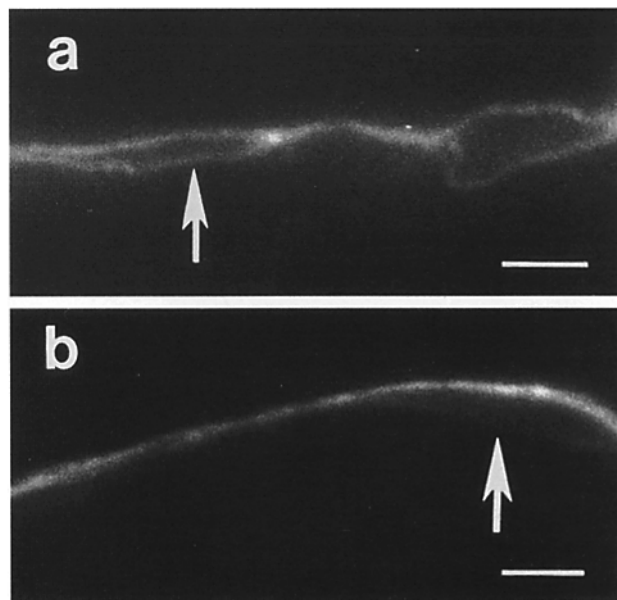


Figure 8. Distribution of adhesion molecules on the apical and basal surfaces of umbilical vein endothelium in situ. mAb against ICAM-1 (*a*) or VCAM-1 (*b*) was incubated with IL-1-stimulated, intact umbilical cords. Subsequent incubation with TRITC-conjugated rabbit anti-mouse IgG permitted visualization of the locations of these molecules in cross sections of umbilical veins. (Arrows) The basal surface of the venular endothelium. Bar, 5 μ m.

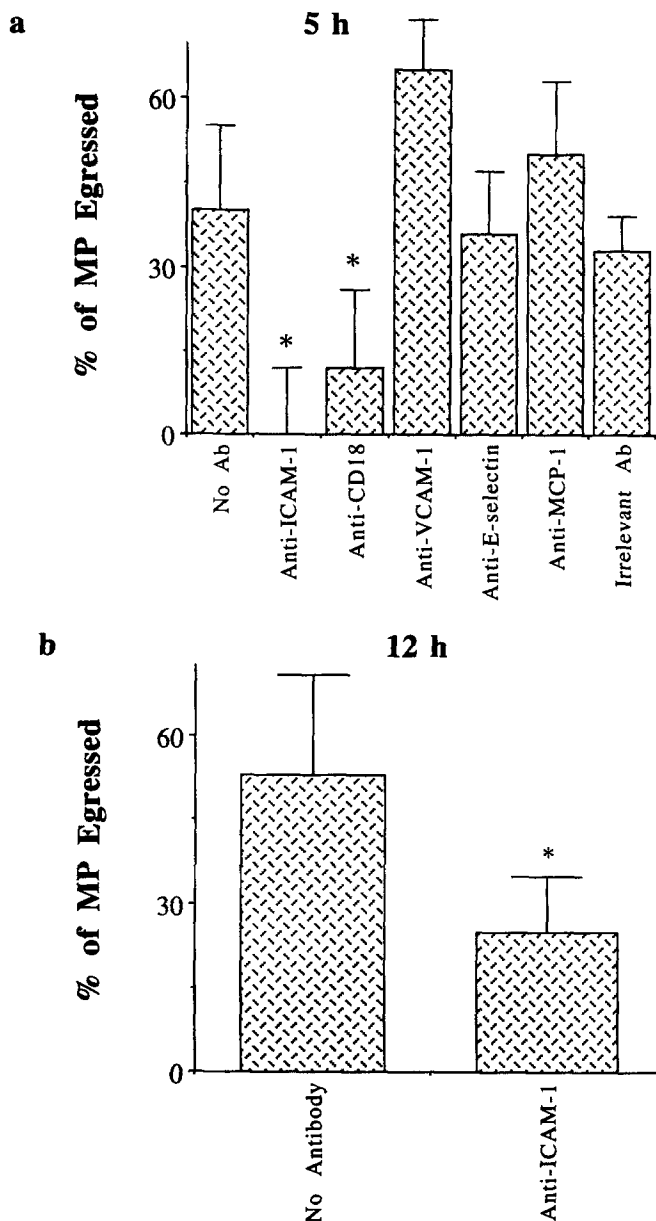


Figure 9. Effect of mAb fragments against adhesion molecules or MCP-1 on egression of MP from HUVEC/amnion cultures. Monocytes were incubated for 2 h with HUVEC/amnion cultures that had been pretreated with IL-1 for 4 h. Cultures were then fixed or rinsed thoroughly in M199 and further incubated for 3 (a) or 10 h (b) in medium lacking Ab or medium containing Fab fragments of an irrelevant mAb (MOPC31c, two experiments, $n = 6$) or mAb fragments against ICAM-1 (R6.5; five experiments at 5 h, $n = 14$; two experiments at 12 h, $n = 6$), E-selectin (H18/7; three experiments, $n = 10$), VCAM-1 (4B9; three experiments, $n = 9$), CD18 (TS1/18; two experiments, $n = 6$), or MCP-1 (S101; two experiments, $n = 6$). Levels of endotoxin in all mAb fragment preparations were <0.06 endotoxin units (EU)/ml at mAb concentrations used in these assays, except MOPC31c, which contained 0.5 EU/ml. In each group, the percentage of MP beneath the HUVEC monolayer was determined and compared to the percentage of MP located beneath the endothelium at 2 h of incubation. (*) Data that are significantly reduced relative to no mAb controls, $p < 0.001$.

If Fab fragments against ICAM-1 inhibited basal to apical migration of MP from HUVEC/amnion cultures by interfering with adhesive contacts between MP and the basal surface of HUVEC, a mAb that blocks binding to CD18, a subunit of the CD11a/CD18 and CD11b/CD18 ligands for ICAM-1 (35, 36) found on MP (37), would also be expected to inhibit egression of MP from IL-1-treated HUVEC/amnion cultures. If, however, anti-ICAM-1 mAb inhibits egression by binding to ICAM-1 on the surface of MP (38), mAb against CD11/CD18 should have no effect. When tested in the assay, anti-CD18 Fab fragments (TS1/18) suppressed egression by an average of 71% at 5 h of incubation (Fig. 9 a). To ensure that anti-CD18 mAb did not simply inhibit the movement of MP through extracellular matrix and thereby impede egression, we tested whether TS1/18 Fab fragments inhibited monocyte binding to and migration within amniotic matrix lacking endothelium in response to 20 nM recombinant human MCP-1 placed beneath the amnion. No difference in the number or depth of penetration into the amnion of monocytes was observed in the presence or absence of 40 $\mu\text{g/ml}$ of anti-CD18 Fab fragments in a 2-h assay (data not shown).

In other control experiments, media from anti-ICAM-1 or anti-CD18-treated HUVEC amnion cultures were collected at the end of the experiments measuring egression and were used in assays measuring transmigration of neutrophils. The media inhibited transmigration of neutrophils in response to FMLP to the same degree as observed previously by fully saturating amounts of these Abs (39), indicating that anti-ICAM-1 and anti-CD18 Fab fragments remained saturating throughout the MP egression assays (data not shown).

Investigation of the Role of MCP-1. We also employed neutralizing mAbs to explore the role of MCP-1 in exit of MP from HUVEC/amnion cultures. MCP-1 has been identified previously in MP/HUVEC cocultures, and it accounts for all of the soluble chemotactic activity for monocytes produced by these cultures (23). Although it is not present in a gradient that would be expected to guide MP across endothelium in the basal to apical direction (23), we aimed to determine whether the chemokinetic activity of MCP-1 (40, 41) might mediate egression. However, anti-MCP-1 Fab fragments did not impede the exit of monocytes from HUVEC/amnion cultures (Fig. 9 a). Control experiments demonstrated that Fab fragments of antibodies to MCP-1 remained saturating during these experiments because enough Ab was present in the medium collected from the cultures at the end of these experiments to inhibit all endogenous chemotactic activity for monocytes in a subsequent blind-well chamber chemotaxis assay (data not shown).

Discussion

In the present study, we have examined the trafficking of MP across endothelial monolayers using an in vitro model of a blood vessel wall. In this model, freshly isolated human

monocytes traverse unstimulated and IL-1-stimulated HUVEC monolayers to enter underlying amniotic connective tissue (20). As demonstrated here, a majority of the transmigrated MP later exit the amniotic tissue by migrating back across the endothelium in the basal to apical direction.

Potential Physiologic Significance of Basal to Apical Transendothelial Migration of MP. Reports of macrophages emerging from atherosclerotic lesions support the possibility that the data presented here are physiologically relevant, since such studies demonstrate that MP can traverse vascular endothelium in the abluminal to luminal direction in vivo. Electron micrographs of arteries from hypercholesterolemic animals strongly imply that monocyte-derived foam cells egress from early atherosclerotic lesions by migrating across intact, overlying endothelium to reenter the circulation (13–15). In addition, degenerate foam cells are found in the circulation, and in liver and spleen, where they are apparently destroyed (13). These observations form the basis of the hypothesis that entrance of monocytes into early atherosclerotic lesions serves the purpose of removing lipid from the lesions via intravasating macrophages, and that inefficient removal of lipid from early atherosclerotic lesions contributes to its progression to more severe stages. Efficient removal of lipid by macrophages, on the other hand, would theoretically lead to regression of the lesion (13).

The data presented here suggest that exodus from tissues by basal to apical migration across endothelium may be a normal activity of MP. Whereas the studies of atherosclerosis strongly suggest that MP can be cleared from tissues by direct reentrance into the bloodstream, they offer no quantitative information about the process. Our data, demonstrating that >80% of MP that migrate into HUVEC/amnion cultures eventually egress by migrating back across the overlying endothelium, suggest that the extent to which MP may use such a mechanism to exit tissues in vivo would be quantitatively significant.

We observed that egression of MP occurred in a diluting environment, as would be found in flowing blood. These results eliminate the possibility that egression of MP from HUVEC/amnion cultures is mediated by a strictly soluble chemoattractant that aberrantly accumulates in the apical compartment of the static cultures. Moreover, the only soluble chemoattractant for monocytes that is detected in MP-HUVEC cocultures is MCP-1 (23), and the data presented here indicate that it does not mediate egression.

In the large volume experiments, only 20% of MP emerged from either unstimulated or IL-1-treated HUVEC/amnion cultures before 24 h of incubation, but as many as 40% exited the cultures by 48 h. These kinetics do not differ greatly from those of clearance of MP from experimentally induced, sterile inflammatory lesions. For example, after instillation of glycogen into rat lungs, the accumulation of mononuclear cells in the pleural cavity is also reduced by ~40% at 48 h relative to a maximal accumulation by 12–18 h (3). It is interesting to note that descriptions of the resolution of inflammatory lesions are remarkably consistent with

the possibility that clearance of at least some of the macrophages occurred by their direct reentrance into the bloodstream:

At 3 days after injection . . . the numbers of mononuclear cells have diminished as compared with 48 hours. In spite of the absence of emigration, many small vessels are surrounded by a zone of mononuclear cells. Almost all of these cells have in varying degrees undergone [differentiative] changes, indicating that some time has elapsed since they left the circulation. . . . Very rarely, one such mononuclear cell is visible trapped within the vessel wall. (5)

Certainly, in vivo studies to determine whether MP traverse endothelium in the abluminal to luminal direction during resolution of acute or chronic inflammation are in order. If physiologic, the implications of our observations may go beyond explaining how MP normally traffic in the body. The mechanism of egression may also lend insight into how tumor cells intravasate during metastasis, illuminate a means by which microorganisms disseminate from organ to organ, and contribute to a more complete understanding of graft rejection. With regard to the latter, Ia⁺ cells migrate from myocardial tissue of donor hearts to the spleens of transplant recipients (42), but the route by which this migration occurs is unknown. It is interesting to note that one subset of Ia⁺ cells, the dendritic cells, is found in the blood in two distinct forms—immature dendritic precursors derived from bone marrow and mature dendritic cells that bear a phenotype resembling dendritic cells found within tissues (43). Perhaps these mature dendritic cells have gained access to the blood by reverse transendothelial migration.

Since migration of MP across vascular endothelium in the abluminal to luminal direction in vivo has yet to be described definitively under circumstances other than within atherosclerotic plaques, alternative explanations for our data must be considered. First, because cultured vascular endothelium may be similar to lymphatic endothelium, we may be modeling how MP exit tissues via the lymphatics. Alternatively, we may have observed basal to apical migration of MP across endothelium in HUVEC/amnion cultures because they lack molecular components that normally retain MP within tissues. Such components could be extracellular matrix molecules or may be produced by interstitial fibroblasts, pericytes, smooth muscle cells, resident macrophages, or other leukocytes.

Mechanism of Egression of MP from HUVEC/Amnion Cultures. The rate at which MP exited HUVEC/amnion cultures decreased when the cultures were placed in a diluting environment, suggesting that a soluble factor(s) mediates the exodus of MP from HUVEC/amnion cultures. In comparison, the initial rate at which MP egress from HUVEC/amnion cultures was increased by pretreatment of the endothelium with IL-1. This increased rate is likely due to upregulation of ICAM-1 on HUVEC in response to IL-1 (44). Supporting this hypothesis, in the presence of anti-ICAM-1 Fab fragments, the kinetics of egression precisely mirror the kinetics of basal to apical transendothelial migration of MP from unstimulated HUVEC/amnion cul-

tures. Beyond accelerating the initial kinetics, the role of ICAM-1 in mediating basal to apical transmigration is unclear. These data suggest that after 5 h of incubation, other molecules, perhaps along with ICAM-1, mediate basal to apical migration of MP across endothelium.

Anti-ICAM-1 mAb appears to block binding of MP to the basal surface of the endothelium, as opposed to interfering with the action of ICAM-1 on the surface of MP themselves. Since CD11/CD18 is present on MP, but not endothelium (36), data indicating that anti-CD18 mAb inhibits basal to apical transendothelial migration of MP imply that MP use CD11/CD18 integrins to ligate ICAM-1 on endothelium while exiting HUVEC/amnion cultures. That anti-CD18 Fab fragments had no effect on the interaction of monocytes with amniotic matrix counters the caveat to this conclusion that the inhibitory effect of anti-CD18 mAb may have been because CD11/CD18 integrins were necessary for movement of MP through matrix. CD11/18 integrins on PMN bind to extracellular matrix molecules *in vitro* (45, 46) and mediate chemotaxis through microporous filters (47), but they do not seem to be essential for MP to bind to extracellular matrix in our cultures.

Complete abrogation of egression for any length of time by addition of Abs to a single adhesion molecule such as ICAM-1 is surprising, since monocytes use multiple adhesive pathways to cross endothelium in the apical to basal direction. Specifically, monocytes can use either CD11/CD18 or very late antigen 4 to migrate across endothelium when initially entering vessel wall constructs (20), and very late antigen 4 and CD11/CD18 can each pair with two or more distinct endothelial ligands in this process (48). These pathways must be blocked simultaneously to produce any inhibitory effects on transmigration. In contrast to our finding that Fab fragments of the anti-ICAM-1 Ab R6.5 completely inhibited exit of MP from HUVEC/amnion cultures, the same preparation of R6.5 had no inhibitory effect on the entrance of monocytes into the cultures (48). These data and the finding that MCP-1 is not essential for egression of MP from HUVEC/amnion cultures, even though this chemoattractant mediates the initial migration of monocytes across HUVEC monolayers (23), clearly illustrate that basal to apical transendothelial migration is not simply a reversal of the same events that promote apical to basal transmigration.

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