Monocyte Long-Term Cultivation on Microvascular Endothelial Cell Monolayers: Morphologic and Phenotypic Characterization and Comparison With Monocytes Cultured on Tissue Culture Plastic

By Ralf R. Schumann, Jürgen van der Bosch, Stephan Rüller, Martin Ernst, and Max Schlaak

Human monocytes were cultured on monolayers of a newly established microvascular endothelial cell strain. As compared to monocytes cultured on plastic, these endothelium-derived monocytes (EDM) showed distinct morphology, higher motility, and different antigen-expression pattern for several surface markers, as detected by cytofluorimetry. The MO-1- and the Leu-M1-marker were maintained on EDMs while they were lost on plastic-cultured cells. The MAX 1-26-termed markers failed to increase on EDMs, in contrast to plastic-cultured monocytes. For seven additional markers, expression after two weeks in vitro was higher on EDMs than on plastic-cultured monocytes. Functionally EDMs showed typical monocyte/macrophage behavior and were easily removable from the culture system for further experimentation. Our data suggest that monocytes cultured on microvascular endothelial cells are maintained for several weeks in a more physiologic state than monocytes cultured on plastic.

We report here on an in vitro system for long-term cocultivation of blood monocytes on microvascular endothelial cell monolayers. Using a set of monoclonal antibodies (MoAbs) and cytofluorimetry, various functional tests, phase contrast, and electronmicroscopy, these endothelium-derived monocytes (EDM) were analyzed in comparison to monocytes/macrophages grown on tissue culture plastic. There is evidence that the endothelium-derived cell comes closer to the in vivo situation of a monocyte/macrophage and expresses more physiologic characteristics than macrophages obtained by presently available methods.

MATERIALS AND METHODS

Establishment of a microvascular endothelial cell strain. A microvascular endothelial cell strain has been established from rabbit lung using a serum-free medium during the initial selection period in vitro, as follows. The lungs of a freshly killed 3-month-old rabbit were removed and washed with several batches of DF containing 2.5 μg of amphotericin B per mL. The outer edges of the lung lobes, which do not contain large blood vessels, were cut off and minced with a multiblade cutting instrument into fragments not exceeding 0.5 to 1 mm. The mince was suspended in 50 mL of DF* containing 2.25 mg collagenase type IV ( Worthington) per milliliter and incubated at 37°C for ten minutes. Then 12 mg dispase, dissolved in 300 μL H2O, were added and incubation at 37°C was continued for another ten minutes. Thereafter 8 mg hyaluronidase, dissolved in 4 mL 150 mg/mL NaCl/10 mmol/L KCl/30 mmol/L MES (pH 4) were added. Five minutes later the suspension was centrifuged for two minutes at 250 g and the pellet was washed twice with 50 mL of DF*. The final pellet was resuspended in 25 mL of serum-free culture medium and seeded 0.5 mL-well in 24-well plates (Nunc, Denmark), which were collagen-coated by preincubation with a 1% solution of Vitrogen 100 (Collagen Corporation) in DF** at 37°C for two hours. Cultures were kept at 37°C in an atmosphere of 5% CO₂ in air. After four days 0.5 mL new medium was added per well and thereafter 0.5 mL medium per well was replaced weekly by new medium.

Cultures exhibiting predominantly outgrowths of endothelial morphology ("cobblenokolike") were detached with 0.2 mL trypsin solution (20 μg trypsin/mL in 0.9% NaCl/10 mmol/L HEPES, pH 7.4) per well. Trypsinization was stopped by adding 1 mL DF** + 5% newborn calf serum per well. After centrifugation at 400 g for

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Submitted November 4, 1987; accepted October 5, 1988.

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0006-4971/89/0303-0001$3.00/0
two minutes the pellet was resuspended in serum-free culture medium and seeded in several collagen-coated wells. Medium in these secondary cultures was renewed as above. Cultures consisting of a seemingly homogenous population of cells with "cobblestone" morphology were selected and passaged further on collagen-coated dishes in DF** containing 5% newborn calf serum.

These endothelial cell cultures (Fig 1) were positive for the von Willebrand factor (vWF) VIII-related antigen, grew in the presence of 5% newborn calf serum with minimum doubling times of approximately one day, and growth was arrested after reaching confluence at cell densities of 7 to 8 × 10^3 cells per cm².

Cell counting was performed by preparing suspensions of cell nuclei, as described previously, followed by counting with a Coulter Counter ZM.

Isolation of human monocytes. Human mononuclear cells were isolated from heparinized venous blood (10 U heparin/mL blood), collected by venipuncture from healthy voluntary donors, according to the method of Bjium and counted with the Coulter Channelizer 256.

The endothelial cell cultures were used 1 week after the last trypsin passage, when they had reached confluency and showed uniform cobblestone morphology. These cultures had obtained their last medium renewal three days before and were washed now twice with warm DF**. Then mononuclear cells suspended in DF** (6 × 10⁶ cells per mL) were added, 0.5 mL per well, and the cultures were incubated for two hours at 37°C in 5% CO₂ to allow attachment of adherent cells. Following adherence, nonadherent cells were removed by rocking of the cells was performed as reported elsewhere; controls were selected for fibroblastic cells was observed, and it was not possible to select stable cell populations of endothelial morphology in serum-containing media.

**Materials.** From Sigma Chemical Co, St Louis, were amphotericin B, hyaluronidase from bovine testes, insulin from bovine pancreas, human transferrin, sodium selenite, triiodothyronine, 2-amino ethanol, hydrocortisone, phosphatidylcholine from egg yolk, cholesterol, α-naphthylesterase kit; from Serva Biochemicals, Heidelberg, West Germany were HEPES, morpholinoethane sulfonic acid (MES), and Triton X-100; from Gibco, Grand Island, NY were DMEM, RPMI 1640, Ham's F 12, newborn calf serum, endothelial mitogen, epidermal growth factor (culture grade); from Boehringer, Mannheim were fetal calf serum, trypsin, medium BM 86; from Merck, Darmstadt, West Germany were ascorbic acid, glutathione, and mercaptoethanol; from Fluka, Switzerland were carmine red; from Jackson ImmunoResearch, Avondale, PA were goat anti-mouse IgG + IgM F (ab)₂-fragment; from Biochrom, Berlin were Ficoll-density 1.077 separation medium; from Nunc, Denmark were culture tubes, tissue culture plastic material; from Becton Dickinson, Mountain View, CA were MoAbs anti-Leu-M₁, anti-Leu-M₂, anti-Leu-M₃, anti-CR-1, anti-CR-2, anti-HLA-DR and antitransferrin receptor; from Coulter, Hialeah, FL were anti-Mo-1 and anti-Mo-2 MoAbs; from Biotest, Dreieich, West Germany were clonab-IL-2-receptor MoAb.

**RESULTS**

Establishment of a microvascular endothelial cell strain from rabbit lung. The enzymatic disintegration of rabbit-lung tissue by means of sequential addition of collagenase, dispase, and hyaluronidase as described in "Methods" is based on earlier experience concerning the disintegration of tumor tissue with these enzymes for tissue culture purposes. This procedure yields a suspension of small cellular aggregates, which attach to the collagen-coated culture substrate. In serum-containing media, extensive outgrowth of fibroblastic cells was observed, and it was not possible to select stable cell populations of endothelial morphology in

Fig 1. Newly established microvascular endothelial cell (MVEC) strain from rabbit lung. Approximately 30 times passed. (A) Unfixed, phase-contrast microscopy (PCM); original magnification x112. (B) Fixed with methanol, PCM, original magnification x200, nuclei and nucleoli visible.
such media. Therefore experiments based on our own and others’ experience with serum-free media were undertaken to avoid overgrowth by fibroblasts during the early phase of establishment. The medium, which finally led to the selection of the endothelial cell strain used in the present work, did not allow continuous propagation at endothelial cells in vitro, but it prevented efficient overgrowth by fibroblasts. Cultures exhibiting predominantly endothelial (cobblestone) morphology were selected and could be passed a few times in this medium without losing their proliferative capacity. At each passage parallel cultures in serum-containing media were derived, which finally gave rise to the endothelial cell strain used in the present work. This strain expressed factor VIII-related antigen on virtually 100% of the cells and was completely homogenous by morphological criteria.

Changes of monocyte morphology induced by cultivation on rabbit-lung microvascular endothelial cells. In Fig 1 the microvascular endothelial cell strain (MVEC) used in the present work is shown. Peripheral blood MNCs of healthy donors can be shown by size distribution analysis to consist of two subpopulations with average cell diameters of 7.3 and 9.5 μm. The larger cells are comprised of 95% monocytes. When freshly prepared MNCs are incubated in vitro on a monolayer of MVECs for two hours at 37°C, the fraction of large cells binds preferentially to the MVECs, whereas a high percentage of the small cells can be washed off (Fig 2).

Following the adherence period, fenestrations of more or less circular shape were observed frequently in the MVEC monolayer. These fenestrations were due to transient retractions of MVECs, generating cell-free areas with diameters of two to five cells. Monocytes were rarely observed in these areas, which were covered again by MVECs within 12 hours.

During the first 24 hours monocytes cultivated on endothelium did not spread as extensively as on plastic. Time-lapse videomicroscopy revealed that the monocytes were much more mobile on the endothelial cell monolayer than on tissue-culture plastic, crawling frequently along the “grooves” between the cells of the MVEC cobblestone monolayer and exhibiting amoeboid shapes with high pseudopodial activity.

During the following days, migrating EDM’s started to form three-dimensional aggregates of spherical and ellipsoid shape. The cells of these aggregates were generally less firmly attached to the MVEC-monolayer than the migrating
Fig 4. Aggregates of EDMs were examined by electronmicroscopy. (A) Cell membranes of six EDMs showing their close contact, original magnification ×3,400. Three nuclei and many vesicles are visible. (B) Higher magnification (original magnification ×18,000) reveals extensive interdigitations of cell membranes in an unique way and large numbers of small vesicles accumulating close to the cell membranes (arrows).

After 1 week large central areas of endothelium were covered by macrophage aggregates. In contrast, monocytes seeded on plastic had formed at the same time an epithelioid-cell–like monolayer with frequently interspersed multinucleated giant cells, which were never observed in EDMs. The cells were large, totally spread, and in part strongly elongated (Fig 3).

Electronmicroscopic examination of the EDMs revealed extensive cell membrane interdigitations between neighboring cells in the aggregates with large numbers of vesicles accumulating at the membranes (Fig 4).

Neither conditioned medium from endothelial cell cultures nor monolayers of fibroblasts from human placenta could mimic the effects of MVEC on macrophage development. In contrast, high monocyte-seeding densities could substitute for MVECs to a limited extent, resulting also in the formation of macrophage aggregation.

EDMs were easily removed from the underlying endothelium by gentle pipetting. When these cells were transferred to plastic surfaces, the aggregates attached and started to spread (Fig 5A), like the monocytes cultured on plastic for the whole period of time. When single-cell suspensions were prepared from EDM aggregates by pipetting and reseeded on MVEC monolayers, macrophages started migrating and reaggregating within 24 hours. EDMs have been kept for up to 16 weeks on the MVEC. They grew larger in size, after 2 weeks became slightly granulated, and sometimes showed vacuoles.

Fig 5. (A) EDMs, if taken off the MVEC layer, adhered to tissue-culture plastic plates and developed like plastic-grown cells. Original magnification ×500. (B) If seeded as single-cell suspension on plastic, EDMs ingest carmine-red, exhibiting the typical “halo,” free of particles, around the cells. Original magnification ×320.

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typic characterization of EDMs was performed:

Although EDMs after a few days of cocultivation did no longer adhere firmly to the MVEC monolayers, they still had the ability to attach and spread on tissue-culture plastic, exhibiting typical macrophage morphology. Ninety-five percent of EDMs transferred on plastic ingested carmine-red particles (Fig 5B). The MoAb Mo-1 is found on human monocytes/macrophages. With indirect immune fluorescence it could be demonstrated that in cocultures of monocytes with MVECs, the cell aggregates consist exclusively of Mo-1-positive cells, whereas the MVEC-monolayer does not bind Mo-1 antibody. Additionally EDMs, transferred to glass coverslips and stained for the monocyte-typical–enzyme-unspecific esterase, consisted of 98% positive cells.

Cytofluorimetric comparison of surface markers on EDMs, macrophages grown on plastic, and freshly isolated monocytes. The following four cell types were studied regarding their expression of surface markers:

1. The monocyte fraction in freshly isolated MNCs as determined by flow cytometric analysis (MNM).
2. Freshly isolated monocytes separated by 1-hour plastic adherence (ADM).
3. Monocytes cultured on endothelial cell monolayers at a density of \( 4 \times 10^4 \) cells/cm\(^2\) (EDM).
4. Monocytes cultured on tissue-culture plastic at a density of \( 4 \times 10^6 \) cells/cm\(^2\) (LDM) and at a density of \( 1.6 \times 10^5 \) cells/cm\(^2\) (HDM).

The cultivated cells were collected after 1 and 2 weeks of in vitro incubation. Single-cell suspensions were prepared of each cell type, and staining with 16 different MoAbs was performed, as described in "Materials and Methods," and subjected to cytofluorographic analysis.

In Fig 6 data on the percentage of positive cells (open bars) and on the mean fluorescence per cell (hatched bars) are compiled for 16 different MoAbs as well as on HDMs and EDMs, starting from very low levels. This increase was observed predominantly during the second week of in vitro cultivation (29% to 60% positive, MF 52-167). Again the EDMs behaved differently: no binding of MAX-1 was observed after 2 weeks. MAX-11 and MAX-24 binding to EDMs was only 20% to 50% of that to LDMs and HDMs; the MAX-26 antibody binding was slightly higher but still reduced in EDMs as compared to the plastic-grown cells.

4. The Leu-M3 antigen is strongly expressed on freshly isolated monocytes (71% positive, MF 161) and can be kept constant or increased if the monocytes are cultivated on plastic, especially if seeded at high densities. If seeded on endothelium, the monocytes showed a decrease after 1 week (36% positive, MF 145), which was partly restored after 2 weeks (41% positive, MF 194), but by far did not reach the levels of LDMs or HDMs.

5. During the first week in vitro, HLA-DR expression increases on HDMs by a factor of 3, whereas on LDMs it decreases by about 30%. At the same time EDMs exhibit a 1.5-fold increase of HLA-DR expression. At the end of the second week in vitro HDMs have lost most of the surface marker gained during the first week, and LDMs exhibit an increased expression, which is about the same as on HDMs at this point of time. EDMs now exhibit the highest HLA-DR labeling.

6. A loss of antigen expression under all culture conditions was detected with the Leu-M2 and the CR-1 marker. However, EDMs started re-expressing CR-1 during the second week in vitro. Neither on freshly isolated nor on cultured cells was expression of MAX-2, MAX-3, or CR-2 detected, with the exception of CR-2 on EDMs, which increased slowly during the 2 weeks of in vitro observation.

Clear differences in antigen expression were observed comparing the MNNs and the ADMs for Leu-M1, transferrin receptor, and MAX-11. With these antibodies a higher percentage of positive cells was detected in ADMs, whereas MO-2 was lost after one hour of adherence.

None of the long-term cultured monocytes (EDM, LDM,
**MONOCYTE-MICROVASCULAR ENDOTHELIUM-COCULTURE**

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Fig 6. Development of expression of 16 different surface markers on cultured human monocytes. Open bars: Percentage of positive cells. Hatched bars: Mean fluorescence per cell (arbitrary units). The studied cells from above to below are: MNM, the monocytic subset of MNCs, detected by flow cytometry (light scattering); ADM, 1-h-plastic-adherence isolated monocytes; EDM, monocytes cultured on endothelium for 1 and 2 weeks; LDM/HDM, monocytes cultured on tissue-culture plastic at low/high densities. Controls (stained without the first antibody) were subtracted from the values before entering this diagram. * - not measured.

HDM) expressed the pan-T-cell marker T11 (data not shown).

**DISCUSSION**

We have developed a new cocultivation system in which, starting from human peripheral blood monocytes, cells develop on microvascular endothelial cell monolayers, which show distinct morphology and antigen expression as compared to plastic-cultured macrophages. The endothelial cell strain used for this purpose was derived from rabbit-lung microvasculature. It grew with stable rates and saturation densities during seemingly unlimited in vitro passages and was therefore preferred to endothelial cells from human tissues, which could not be handled equally well. In some respects the EDMs resemble in vivo monocytes/macrophages, and it is presumed that for the development and culture of monocytes, the endothelial cell is of benefit.

Monocytes, if seeded on plastic, usually adhere strongly to the substrate and are heavily immobilized, as documented by time-lapse videomicroscopy. After 1 week they are spread totally, as seen in our experiments and as described by others. In contrast, EDMs remain in a state of high motility. They move across the endothelium and toward each other and form aggregates. Their migration rate is approximately 240 times higher than that of LDMs and HDMs, as evaluated in a video motility essay.

The culture substrate being important for monocyte cultivation and differentiation has been described by others.

Previous studies used glass, plastic, teflon, collagen, and other matrices. The development of monocytes on endothelial cell monolayers seems to involve specific interactions between these cells and is not simply due to lowered adhesiveness of the substrate: neither fibroblasts nor collagen substrate could substitute for endothelial cells, whereas bovine aortic endothelial cells and high-density monocytes could. This suggests specific interactions between endothelial cells and monocytes, which can be mimicked in part by monocyte-monocyte interaction but not by fibroblast-monocyte contacts (unpublished observations). Microvascular endothelium might be an especially interesting substrate for monocyte development, since contact and interactions between monocytes/macrophages on the one side and MVECs on the other side occur frequently in vivo (eg, diapedesis of monocytes through the vessel wall).
In this respect the following data on EDMs deserve special attention: the MO-1 marker is kept at high levels on EDMs, whereas it decreases to insignificant levels on LDMs and HDMs. This decrease on plastic-cultured monocytes has been observed by others as well.\(^\text{4,6,7}\) The MO-1 surface glycoprotein, which is the C3bi receptor, is important for several monocyte functions like adherence, binding, and ingestion of serum-opsonized particles, zymosan-induced degranulation, and superoxide generation.\(^\text{46}\) On peripheral blood monocytes as well as on various types of human tissue macrophages, this antigen is found to be expressed significantly,\(^\text{49,51}\) and the equivalent mouse antigen is found on mouse monocytes and tissue macrophages.\(^\text{52}\) Permanent deficiency of this antigen in animals as well as in humans leads to recurrent bacterial infections, delayed wound healing, and severe leukocytosis, as case reports reveal.\(^\text{48,53,54}\) Increased or excessive MO-1 expression is reported to be associated with insulin-dependent diabetes and is suspected of playing a role in the chronic diabetic vascular disorder due to increased adhesiveness of monocytes.\(^\text{55}\) Thus the disturbance of MO-1 expression seems to signal pathologic conditions in vivo and probably indicates inappropriate culture conditions in vitro. In this context is is interesting that γ-IFN-induced loss of MO-1 antigen in cultured monocytes can be restored by the addition of fibronectin\(^\text{55,56}\) and that in the presence of MVECs loss of MO-1 antigen is prevented, as demonstrated in the present work.

C3b and C3d receptors are further examples of maintenance of physiologically meaningful functions on EDMs, which are lost on plastic-cultured macrophages, as reported by others.\(^\text{57}\) Another marker found on blood monocytes and on some tissue macrophages in vivo\(^\text{58,59}\) is Leu-M1. In our experiments this marker was retained on EDMs, whereas it was lost on LDMs and HDMs.

For the new set of MoAbs, termed MAX 1-26, which are reported to increase on monocytes cultured on plastic or teflon,\(^\text{58,60}\) in the present work only four of the six markers tested were induced on plastic-cultured monocytes. However, no expression was observed on EDMs. These results again suggest a higher degree of similarity between EDMs and monocytes than between plastic-cultured cells and monocytes.

In recent studies transferrin and IL-2 receptors were shown to be expressed during in vitro culture of monocytes.\(^\text{61,62}\) Little is known about the expression of these two markers on monocytes/macrophages in vivo. We found in our experiments a markedly stronger increase of these two receptors on EDMs than on LDMs and HDMs. The de novo expression of these receptors is usually discussed as an indication of macrophage activation. Since both these receptors are also expressed predominantly on proliferative active cells, their expression on macrophages could be also interpreted as signaling a state of proliferation competence (manuscript with additional data in preparation).

In conclusion, it can be stated that the long-term cocultivation of human monocytes on microvascular endothelial cells leads to the development of a cell population, which is distinguished from plastic-cultured macrophages by several markers, which are expressed on EDMs rather like on freshly isolated monocytes in the nonadherent state or on tissue macrophages. It is thought that this kind of culture system provides a more physiologic microenvironment for monocyte/macrophage development than the conventional culture techniques. It avoids contact with plastic and leaves the cells in an "intravascularlike" microenvironment. Such systems should be especially useful for the investigation of the human monocyte/macrophage, which is not routinely accessible to the researcher in the multiple physiologic and pathogenic conditions that are characterized by its presence. The introduction of human endothelial cells from various tissues and the inclusion of cytokines, which are known to be involved in the interaction of monocytes and endothelium, will certainly yield new insights into monocyte/macrophage physiology.

ACKNOWLEDGMENT

We are very grateful to Dr. Jürgen Galle for the performance of the electronmicroscopic studies and for helpful discussions. Our thanks are also due to Dr. H.W.L. Ziegler-Heitbrock, München, for carefully and critically reading the script and for fruitful discussion and encouragement. We thank Dr. Reinhard Andreassen, Freiburg, for having made available to us the MoAbs MAX 1-26. Furthermore we are indebted to Inka Kronenbitter and Renate Bergmann for excellent and devoted technical assistance and to Renate Mohr and Frauke Richter for preparing the typescript and the figures in this manuscript.

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