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Regulation of human monocyte adherence by granulocyte–macrophage colony-stimulating factor

(endothelium/inflammation/atheroma/growth factors)

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ABSTRACT **Recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) was found to increase the adherence of purified peripheral blood monocytes to plastic surfaces and to monolayers of human umbilical vein endothelial cells. With plastic surfaces as a model 9-hr culture with GM-CSF was necessary for enhancement, and maximum levels were obtained after 24-hr stimulation. GM-CSF-stimulated adherence must require new RNA and protein synthesis because actinomycin D and cycloheximide abolished existing adherence and prevented further monocyte attachment. Interestingly, shorter incubations (1–2 hr) with cycloheximide increased adherence, suggesting a labile inhibitor. Formaldehyde fixation of monocytes but not of human vein endothelial cells abolished adherence, indicating the need for actively metabolizing monocytes. Thus, a hemopoietic growth factor, responsible for the proliferation and differentiation of monocytes, can also alter their adhesive characteristics. These observations may have important implications in pathological situations and in the *in vivo* use of GM-CSF.**

Adherence of human blood monocytes to vascular surfaces is an essential component of several pathological processes, including inflammation and atheroma. Freshly isolated monocytes show a high level of adhesion to endothelial monolayers (1, 2) and plastic surfaces (3). After adhering to endothelium, monocytes rapidly migrate through cellular junctions to the subendothelial space (4, 5). Just as neutrophils can be stimulated to adhere to different surfaces, monocytes can be treated with phorbol esters (2) or chemotactic peptides (6) to stimulate adherence; furthermore, interleukin 1 (7) and tumor necrosis factor α (TNF-α; J.R.G. and M.A.V., unpublished observations) induce in endothelium increased adhesiveness for monocytes.

Recently we showed that the hemopoietic growth factor granulocyte–macrophage colony-stimulating factor (GM-CSF) prolongs the survival of monocytes in culture (32), and others have shown that GM-CSF activates the capacity of monocytes to kill tumor targets (8–10), stimulates monokine production (11, 12) and morphological changes (13). These observations suggested that GM-CSF might also activate monocytes in the circulatory system and lead to increased adhesion to endothelium. Such a phenomenon would explain the observation that GM-CSF given i.v. to humans leads to localized phlebitis (14) and given chronically to mice leads to major abnormalities in which monocyte infiltration of tissues is the dominant pathological outcome (15).

In this communication we show that the adhesion of human monocytes is regulated by the hemopoietic cytokine GM-CSF. This enhanced adhesiveness is evident on plastic surface as well as on endothelial cells and was seen at low concentrations of GM-CSF. Our observations suggest that GM-CSF may regulate monocyte adhesion to vascular surfaces and have an important role in related pathological phenomena.

MATERIALS AND METHODS

Cell Preparations. Peripheral blood from normal patients was obtained from the Red Cross Transfusion Service (Adelaide, South Australia). The mononuclear cell fraction was prepared by density-gradient centrifugation on lymphoprep (Nyegaard Oslo), washed twice in Hanks' balanced salt solution (HBSS), and then resuspended in HBSS/0.02% EDTA/0.1% fetal calf serum (Flow Laboratories). The monocytes were further purified by countercurrent elutriation in a Beckman JE-6B elutriator with a Sanderson chamber (16). Rotor speed was 2050 rpm, and flow rate was 12 ml/min. The cells remaining in the chamber after 30 min were collected and washed in RPMI 1640 medium. Cytocentrifuge preparations were stained with Geimsa stain, and only preparations judged to be >90% monocytes were used in these experiments. For monocyte fixation, cells were pelleted and resuspended in 1% formaldehyde solution in phosphate-buffered saline (PBS) and incubated for 15 min at room temperature after which time they were washed in RPMI 1640 medium/10% fetal calf serum.

Human umbilical vein endothelial cells (HUVEC) were obtained by collagenase treatment of umbilical veins. The cells were plated onto gelatin-coated Costar 25-cm^2 flasks, maintained as described (17) and used between 4 and 8 days after culture establishment. For use in adherence assays, cells were harvested by using trypsin/EDTA and replated into the central 60 wells of gelatin-coated flat-bottomed microtiter trays (Nunc) at 2×10^4 cells per well and grown to confluence overnight. For fixation of HUVEC, the monolayers were washed once in PBS, 200 μl of 1% formaldehyde/PBS was added for 15 min at room temperature, and the monolayers were then washed twice in RPMI 1640 medium/2.5% fetal calf serum before use. No change in the morphology or shrinkage of the HUVEC monolayer was evident by light microscopy with this procedure.

Isotopic Labeling of Monocytes. After purification, for some experiments monocytes were labeled with ^{51}Cr by incubation in a 1-ml volume containing 400 μCi of ^{51}Cr-labeled sodium chromate (1 Ci = 37 GBq; Amersham) at 37°C for 45 min with gentle agitation. After incubation, free ^{51}Cr was removed by three washes in RPMI 1640 medium.

Suspension Cultures of Monocytes. In some experiments monocytes were incubated for 24 hr in suspension before measuring adherence. In this case the cells were resuspended in RPMI 1640 medium/10% fetal calf serum at 2.5×10^6 cells per ml and rotated in polypropylene tubes at 37°C either with or without GM-CSF. Cells were then washed once in RPMI

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Abbreviations: HUVEC, human umbilical vein endothelial cell(s); GM-CSF, granulocyte–macrophage colony-stimulating factor; TNF-α, tumor necrosis factor α; PBS, phosphate-buffered saline.

1640 medium, cell counts were performed, and the cells were used immediately. With this procedure we routinely observed only 10–20% reduction in cell numbers with no differences seen between groups incubated with or without GM-CSF. The recovered cells were >99% viable as judged by eosin dye exclusion.

Measurement of Adherence. Monocyte adherence to plastic and HUVEC was measured by either of two methods. (*i*) Uptake of the vital stain rose bengal, as described (18) or (*ii*) labeling of monocytes with ^{51}Cr. For each method, 3×10^5 cells were plated into microtiter wells either with or without the addition of cell stimulators in a total volume of 100 μl. The cells were incubated at 37°C and 5% CO_2 for the times indicated in each experiment.

For assessing adherence level by staining, the supernatant was removed, and the cells were stained with rose bengal (0.25% in PBS, pH 7.3) for 20 min. The nonadherent cells and the excess stain were removed by aspiration and four washes in RPMI 1640 medium/10% fetal calf serum. One hundred microliters of ethanol/PBS (1:1) was added to each well, and the OD at 570 nm was read on an ELISA reader (Titretek) 60 min later. The adherence level is expressed as OD_{570} units. For attachment of monocytes to endothelium, the OD_{570} value of wells containing only HUVEC is subtracted from the value of wells containing monocytes and HUVEC to give the adherence value: Adherence (OD_{570}) = OD_{570} value of (monocytes + HUVEC) − OD_{570} with only HUVEC.

For isotopic measurement of monocyte adherence, after incubation in the microtiter wells, aliquots of supernatants were counted to measure spontaneous ^{51}Cr release. Remaining supernatants were then removed by aspiration, and the cells were washed three times to remove nonattached monocytes. The cells were lysed by adding lysis buffer containing 10 mM Tris·HCl, 0.15 M NaCl, and 1% Nonidet P-40. The contents of each well was then counted in a Packard γ counter. Percent adherence was determined as follows: % adherence = (cpm of ^{51}Cr in lysate/cpm of total-cell-associated ^{51}Cr) × 100, where total-cell-associated ^{51}Cr is calculated as total ^{51}Cr added minus ^{51}Cr spontaneously released in cpm.

Cytokines and Antibodies. Recombinant human TNF-α (lot 3056-55, 2.5×10^7 units/mg) was supplied by Genentech. Recombinant human GM-CSF (purified from COS cell supernatants, batch COO4D1801) was supplied by Genetics Institute (Boston). Both preparations were tested for endotoxin by the limulus amoebocyte lysate assay and found to contain <0.1 enzyme unit per ml. Polyclonal sheep anti-GM-CSF (S7, 5/8/87) and rabbit anti-interleukin 3 (R165, 10/21/87) were provided by Genetics Institute. These antibodies have been shown in other assays to have specific neutralizing activity.

RESULTS

GM-CSF, but Not TNF-α, Enhance Monocyte Adherence to Plastic. Elutriated monocytes adhere avidly to plastic for several hours, but this adhesiveness rapidly disappears with time. After 18- to 24-hr of incubation most cells are nonadhesive. To test the ability of cytokines to affect adhesion, elutriated monocytes were aliquoted into 96-well plastic plates and incubated for 18 hr with graded concentrations of cytokines. Fig. 1*A* shows that GM-CSF, but not TNF-α (up to 100 units/ml), caused a dose-dependent increase in adhesion. This phenomenon was seen whether adhesion was measured by the dye-uptake method or the radioisotopic method and was significantly inhibited by specific antiserum against GM-CSF but not by control antiserum (Fig. 1*B*).

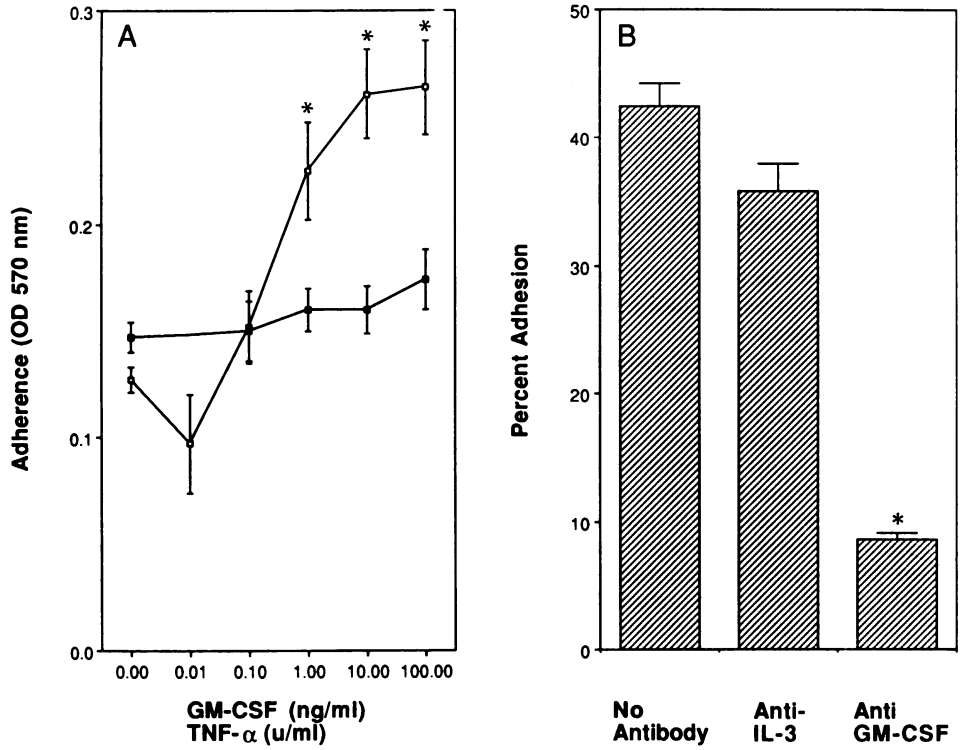

FIG. 1. (*A*) Effect of various concentrations of GM-CSF (□) and TNF-α (■) on monocyte adherence to plastic. The cytokines were added at the beginning of culture, and adherence was measured 24 hr later. Data represent the means (± SEM) of 11 experiments with GM-CSF and 5 experiments with TNF-α; triplicate determinations were made for each experimental group. *, $P < 0.0005$ (by unpaired Student's *t* test on experimental vs. unstimulated groups). (*B*) Effect of antiserum to GM-CSF and to interleukin 3 (IL-3) on GM-CSF-stimulated monocyte adherence to plastic determined by the radioisotopic method. Antiserum, at final dilution of 1:1000, and GM-CSF at 1 ng/ml were premixed in wells for 30 min at room temperature before adding monocytes. Adherence was measured 24 hr later. Each point represents the mean (± SEM) of six determinations in a representative experiment. *, $P < 0.0001$ compared with group lacking antibody.

Experiments done in parallel with the rose bengal and radioisotopic methods for assessment of adherence showed similar levels of increase in monocyte adherence after GM-CSF stimulation. For 14 experiments done with the ^{51}Cr assay, basal adherence was 12.8 ± 2.9% (mean ± SEM); after stimulation with GM-CSF at 10 ng/ml, adherence was 43.8 ± 4.9%. Of 11 experiments assayed with rose bengal OD increased from 0.051 ± 0.003 (basal adherence) to 0.189 ± 0.016 with GM-CSF at 10 ng/ml.

Time Course of Induction of Adherence by GM-CSF. Monocytes were cultured for 24 hr in plastic plates to which GM-CSF was added at various times before measuring adherence. Fig. 2*A* shows that maximum adherence was seen when GM-CSF was present for the entire incubation (that is, for 24 hr) and was still effective when added 9, but not 6, hr before assay. In wells not receiving GM-CSF the cells remained fully viable and could be stimulated to adhere by subsequent addition of GM-CSF. In these experiments the cells were incubated without GM-CSF for 24 hr, and the cytokine was added at different concentrations for 12 hr before adherence levels were measured (Fig. 2*B*).

Effect of Inhibitors of Protein or RNA Synthesis on Monocyte Adherence. That protein or RNA synthesis is involved in production of the adhesive state was tested by adding cycloheximide or actinomycin D. In preliminary experiments the presence during the entire 24-hr incubation period of either agent at 5 μg/ml abolished adherence (data not shown). To examine this inhibition in more detail an experiment was designed during which cycloheximide or actinomycin D at 5 μg/ml was added to ongoing cultures of monocytes after 16–23 hr (that is, for the final 8–1 hr of incubation). Monocyte adhesion was measured at 24 hr. To assess the adhesion level at the time of inhibitor addition, parallel cultures were terminated at some of these times, and adhesion was measured.

GM-CSF-enhanced adhesion was inhibited by actinomycin D in a time-dependent fashion (Fig. 3*A*). Addition of cycloheximide resulted in a biphasic effect—an enhancement when added for the last 2 hr but a strong inhibition when added earlier. Comparison with adhesion levels in groups without inhibitors at various times showed that both agents not only inhibited further adhesion but prevented the maintenance of existing adherence.

The effect of these inhibitors on basal monocyte adherence to plastic was also investigated (Fig. 3*B*). Addition of actinomycin D inhibited basal adhesion when added at any time during the final 8 hr of culture. Cyclohexamide addition again had a biphasic effect: when added at 23 or 22 hr (that is, present for only the final 1–2 hr), a significant increase in adhesion occurred. In contrast, a decrease in adhesion resulted when cycloheximide was added early (for example, at 16 hr). The high levels of basal monocyte adherence at 16 and 18 hr reflect the strong initial adherence to plastic (which forms the basis for many separation procedures for monocyte purification) that declines with time.

Effect of GM-CSF on Adhesion of Monocytes to Endothelium. Monocytes were cultured in suspension for 24 hr with graded concentrations of GM-CSF before measuring adherence to HUVEC. This protocol was chosen to diminish the possibility of immunological reactions between HLA-incompatible donors of monocytes and endothelial cells and the confounding effect of secreted products of either cell during longer incubation. Results of a single experiment (representative of four experiments with similar results) are shown (Fig. 4) and demonstrate that monocyte adherence to confluent monolayers of HUVEC is increased by GM-CSF stimulation. This was a consistent finding and was reflected by similar results in plastic adherence of the same population

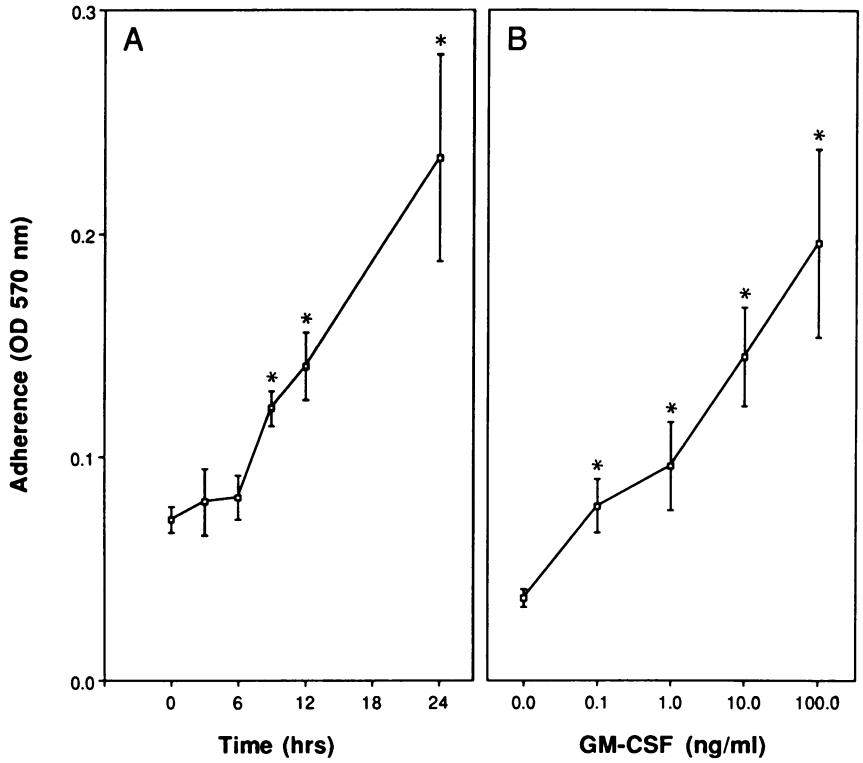

FIG. 2. (*A*) Effect of GM-CSF on monocyte adherence. Monocytes (3×10^5) were added to microtiter wells, and GM-CSF at 100 ng/ml was present for the indicated times. Total incubation time was 24 hr. The mean (± SEM) of at least nine determinations from three experiments with triplicate determinations at each point is given. *, $P < 0.0001$ compared with the group not receiving GM-CSF. (*B*) Effect of culturing monocytes for 24 hr without cytokine on their ability to respond to GM-CSF. Monocytes were cultured in microtiter wells for 24 hr before adding various concentrations of GM-CSF; adherence was measured 12 hr later. Each point represents the mean of at least six determinations from two experiments (± SEM). *, $P < 0.0008$ compared with the group not receiving GM-CSF.

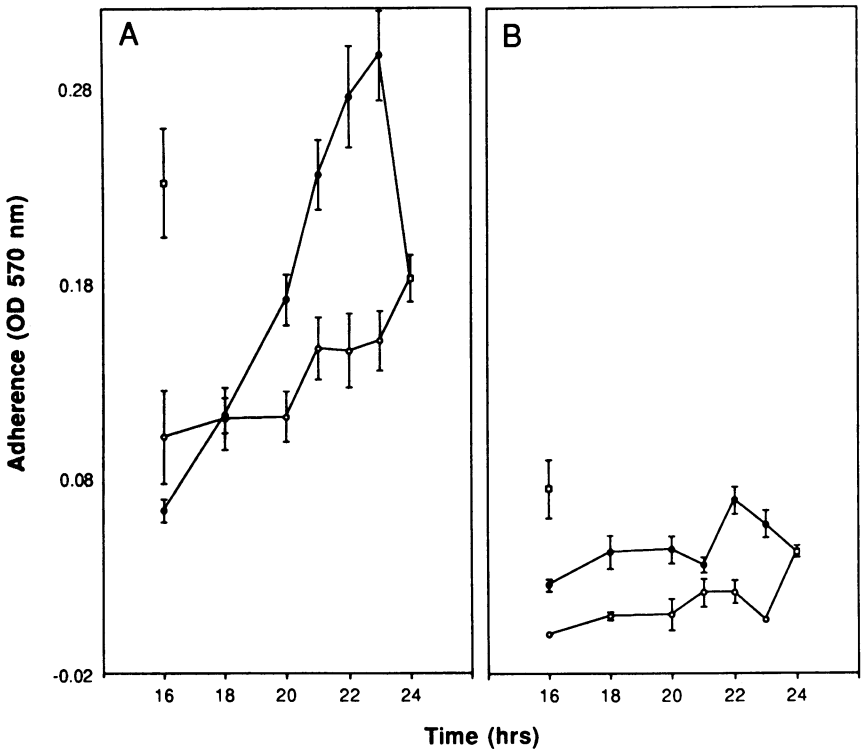

FIG. 3. (*A*) Effect of cycloheximide (●) or actinomycin D (○) (5 μg/ml) on GM-CSF-stimulated adherence of monocytes. At indicated times after culture initiation the drugs were added, and adherence was assayed at 24 hr. Results represent the mean (± SEM) of two to seven experiments done in triplicate. Actinomycin D inhibited adhesion when added at 16, 18, and 20 hr ($P < 0.05$, $P < 0.02$, and $P < 0.04$, respectively). Cycloheximide enhanced adhesion when added at 22 and 23 hr ($P < 0.03$ and $P < 0.0001$, respectively) and inhibited at 16 hr and 18 hr ($P < 0.002$ and $P < 0.02$, respectively). The open square at 16 hr represents adhesion in parallel experiments without any inhibitor that were terminated at that time. (*B*) Effect of cycloheximide (●) or actinomycin D (○) (5 μg/ml) on basal adherence of monocytes. At indicated times drugs were added to the incubation, and adherence was assayed at 24 hr. Actinomycin D inhibited adhesion when added at 16, 18, and 23 hr ($P < 0.008$, $P < 0.005$, and $P < 0.02$, respectively). Cycloheximide increased adhesion when added at 22 and 23 hr ($P < 0.0007$ and $P < 0.04$, respectively). The open square at 16 hr represents adhesion of parallel experiments without inhibitor that were terminated at that time.

of monocytes, although the relative attachment levels to HUVEC and plastic varied (Table 1).

DISCUSSION

Monocytes subserve important functions in chronic inflammation, antigen presentation, and the formation of atherosclerotic lesions. Understanding of the mode of migration of monocytes from the circulation to tissue sites is essential in investigations of monocyte biology. This migration, in turn, depends on how the adhesion to vascular tissues, in particular to endothelium, is regulated.

Our central observation is that a hemopoietic cytokine, GM-CSF, involved in stimulating the proliferation and differentiation of monocytes, also stimulates their adhesiveness to plastic or endothelial surfaces. Small concentrations of GM-CSF (0.1–1.0 ng/ml) were active, and although the level of stimulation varied from donor to donor, the phenomenon was significant and reproducible.

Monocyte adhesion involves both CDw18-dependent and CDw18-independent components (2) and differs depending on whether it is measured with monolayers or cells in suspension (19). Our observations make the additional point that an actively metabolizing monocyte is essential to attaining and maintaining the adhesive state. Fixation (Fig. 4) and inhibition of RNA or protein synthesis (Fig. 3) inhibit mono-

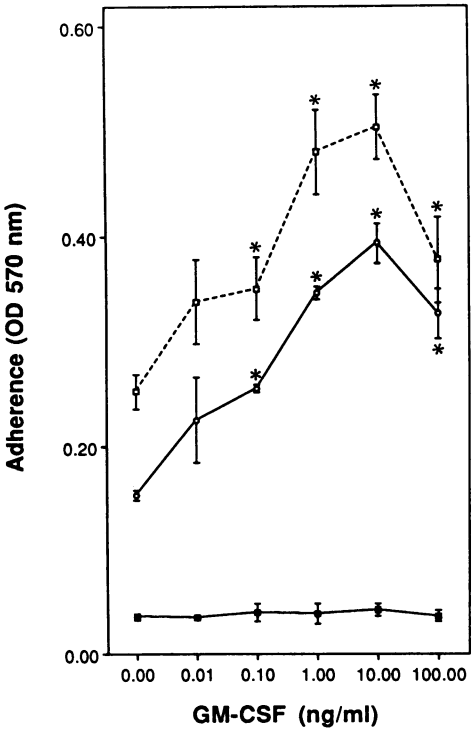

FIG. 4. Effect of GM-CSF on monocyte adherence to endothelial monolayers. Monocytes incubated in suspension cultures for 24 hr with graded doses of GM-CSF were added to unfixed HUVEC (---) or fixed HUVEC (—), and adherence was measured after 45 min. Monocytes were fixed before addition to HUVEC (■). The mean (± SEM) of triplicate determinations from a single experiment is given. *, $P = 0.05$ or less compared with the group not receiving GM-CSF.

Table 1. Monocyte attachment to endothelium and to plastic

Surface	Adherence		*P*
	Unstimulated	GM-CSF stimulated	
^{51}Cr assay*			
HUVEC	20.9 ± 1.4	33.4 ± 1.5	0.00001
Plastic	11.4 ± 2.5	24.0 ± 4.5	0.03
Rose bengal stain†			
HUVEC	0.376 ± 0.030	0.529 ± 0.051	0.02
Plastic	0.373 ± 0.046	0.530 ± 0.046	0.03

Monocytes were incubated in suspension for 24 hr either with or without GM-CSF (10 ng/ml). Cells were washed, counted, and added to either HUVEC monolayers or to plastic for 45 min, at which time adherence was measured. *P* values represent the difference between the unstimulated and stimulated groups.

*% adherence (mean ± SEM) of three experiments with triplicate determinations in each group for each experiment with ^{51}Cr-labeled monocytes.

†OD_{570} (mean ± SEM) of three different experiments with triplicate determinations in each group for each experiment; attachment level was measured by stain.

cyte adhesion. Interestingly, the same considerations do not appear to apply to the endothelium, which supports strong adhesion whether in the fixed or unfixed state.

The increase in adhesion upon stimulation with GM-CSF may be the result of upregulation of the surface expression of adhesion molecules, such as p150,95, which are involved in monocyte adherence (3). Alternatively, our data could be interpreted in terms of the observation of Altieri and Edgington (20), who demonstrate functionally different conformational states (conformers) in adhesive molecules. The trigger for this change (perhaps following upregulation of adhesion molecules) may be passive (fixed endothelium or plastic) but needs actively metabolizing monocytes. Our studies of GM-CSF-stimulated monocyte adherence to HUVEC and plastic surfaces show that attachment is, at least in part, mediated by common mechanisms (M.J.E., M.A.V., J.R.G., and A.F.L., unpublished work).

An interesting observation emerged in the cycloheximide experiments, where a transient hyperadhesiveness in unstimulated monocytes was seen (Fig. 3*B*); this phenomenon probably accounted for the biphasic effect on GM-CSF-stimulated adherence (Fig. 3*A*). A similar phenomenon has been seen by us (21) and others (22) in treating endothelial cells with cycloheximide for 4–6 hr. The effects of cycloheximide on monocytes may be most easily explained by postulating the existence of a protein that inhibits adhesion and is rapidly turned over. This putative protein could be central in ensuring monocyte nonadhesiveness *in vivo*; identification of such a protein would be of some interest.

Given that migration through endothelium follows adhesion (5), our results have relevance to several disease states: (*i*) In experimental models where GM-CSF is chronically in excess, serious pathology develops; a constant feature of this pathology is extensive tissue invasion by monocytes (15). (*ii*) Because monocytes are a rich source of TNF-α and GM-CSF can increase TNF-α mRNA and prime for TNF-α secretion in human monocytes (11), the GM-CSF-induced adhesive state may signal the beginning of a vicious cycle. Adherent monocytes may locally elaborate TNF-α, which, in turn, stimulates endothelial cells to develop a proadhesive and prothrombotic phenotype (23–25) to support stronger adherence of monocytes and to secrete GM-CSF (26). Indeed, thrombotic reactions have been seen in short-term use of GM-CSF in humans (27), and excessive monocyte–cell wall interaction may be an important initiating event. Because monocytes can also produce GM-CSF (28), an autocrine mechanism for increases in monocyte adherence could be further postulated. (*iii*) The pathogenesis of atheroma involves monocyte adhesion to arterial endothelium as an initiating event (29, 30). Thereafter, fat-laden monocytes migrate to the subendothelium and become foam cells (31). GM-CSF may play a critical role in initiating or maintaining this process, and genetic heterogeneity in blood levels or responsiveness to GM-CSF, as shown for neutrophils (J.R.G., T. Rand, I. Clark-Lewis, A.F.L., and M.A.V., unpublished work), may be an important determinant in atheroma development.

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