Lack of Effect of Granulocyte-Macrophage and Granulocyte Colony-Stimulating Factors on Cultured Human Endothelial Cells

By K. Yong, H. Cohen, A. Khwaja, H.M. Jones, and D.C. Linch

The hematopoietic growth factors, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), enhance the effector functions of mature myeloid cells, including the interaction with vascular endothelium. We examined the direct effect of recombinant human GM-CSF (rhGM-CSF) and recombinant human G-CSF (rhG-CSF) on the growth and function of cultured human umbilical vein endothelial cells (HUVEC). Endothelial cell growth supplement (EGCS) increased the proliferation of passaged and primary cells by 305% ± 45% (mean ± SEM, n = 5, P < .01) over control cells at 4 days; GM-CSF and G-CSF had no effect. Endothelial cell procoagulant activity was increased after 4-hour incubation with recombinant interleukin-1 β (IL-1β) 10 U/mL and recombinant tumor necrosis factor (TNF) 10 U/mL to 1.721% ± 376% (n = 7, P < .005) and 247% ± 71% (n = 4) of control levels, respectively. γ-Interferon (γ-IFN) 50 U/mL had no direct effect of its own but was able to prime the response to IL-1β. There was no direct or priming effect of GM-CSF (1 ng to 1 μg/mL) on the expression of procoagulant activity in endothelial cells. GM-CSF and G-CSF (1 ng/mL to 1 μg/mL) had no effect on the expression of either tissue plasminogen activator (tPA) or plasminogen activator inhibitor-1 (PAI-1) by endothelial cells. The secretion of tPA by endothelial cells was increased, however, after 24-hour incubation with thrombin 4 U/mL (314% ± 72% of control levels, n = 5, P < .025). The production of PAI-1 was increased by TNF 200 U/mL (241% ± 44% of control, n = 3, P < .005), thrombin 4 U/mL (180% ± 12% of control, n = 5, P < .0005) and IL-1β 10 U/mL (275% ± 44% of controls, n = 5, P < .0005). In four experiments, endothelial cells showed no specific binding of 125I-GM-CSF, whereas peripheral blood (PB) neutrophils demonstrated the presence of 802 ± 78 high-affinity receptors for GM-CSF. Thus, we found no effect of rhGM-CSF or rhG-CSF on the proliferation of HUVEC or the expression of procoagulant and fibrinolytic activities by these cells. These findings are in accordance with the lack of demonstrable receptors for GM-CSF on cultured HUVEC.

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was determined by proliferation assays using a GM-CSF-dependent cell line and by flow cytometric priming assay of the neutrophil respiratory burst. The 3H-GM-CSF specific activity was determined by a combination of enzyme-linked immunosorbent assay (ELISA) (Medgenix Diagnostics, Bucks, England) and biologic assays (described above), which gave comparable results and determination of maximum binding capacity and self-displacement activity as described previously.

Reagents. RPMI 1640, Iscove's modified Dulbecco's medium (IMDM), M-199, fetal calf serum (FCS), penicillin/streptomycin, and phosphate-buffered saline (PBS) were obtained from Gibco Laboratories, Uxbridge, UK. Trypsin/EDTA, fibronectin, and collagenase were obtained from Boehringer Mannheim. 3,4,5-Dimethylthiazol-2-yl2,5-diphenyltetrazolium bromide (MTT) and EGTA were obtained from Sigma. The chromogenic substrate S2222 was obtained from Kabi (Stockholm, Sweden). Factor VII, II, IX, and X concentrates were supplied by Blood Products Laboratory, Elstree, England. Rabbit thromboplastin was obtained from Diagen, UK. Anti-von Willebrand factor monoclonal antibody (vWF MoAb) was obtained from Dako, UK.

Cell cultures. HUVEC were harvested from umbilical cords within 6 hours of delivery by collagenase treatment (0.1%) and grown to confluence on fibronectin-coated (2 μg/cm²) plastic in IMDM or RPMI 1640 with 20% FCS, penicillin/streptomycin (100 U/mL/100 μg/mL), ECGS (25 μg/mL), and heparin (50 μg/mL). Cells were identified by typical cobblestone morphology and granular staining pattern with vWF MoAb. Cultures were passaged after a 2-hour incubation to allow the cells to adhere, the wells were rinsed twice with medium and an MTT assay was performed on half the wells. On the other four wells of each data point, cells were removed by trypsin/EDTA treatment and counted on a Neubauer counting chamber. The number of cells removed from each well was within 5% to 10% of the number seeded. Figure 1 (inset) shows a calibration curve of absorbance units against cell numbers derived from three separate experiments.

Procoagulant assays. Subconfluent HUVEC (passages 1 and 2) were seeded (2 × 10³ cells per well) in 96-well fibronectin-coated microtiter plates. Cytokines were either added at the time of seeding or added to cultures at 50% confluence after 48-hour serum starvation. A standard MTT assay was used to quantify cell numbers. Cells were rinsed twice with medium before being incubated with MTT, 1 mg/mL in medium, 100 μL/well, for 2 hours at 37°C. The incubation solution was aspirated, and 100 μL dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. The change in absorbance was read at 570 nm on an MR 700 Microplate Reader (Dynatech Laboratories).

The validity of this assay as a measure of cell proliferation was tested in two ways. First, the assay was calibrated by seeding known numbers of cells in 96-well tissue culture plates. Eight wells were used for each data point. After a 2-hour incubation to allow the cells to adhere, the wells were rinsed twice with medium and an MTT assay was performed on half the wells. On the other four wells of each data point, cells were removed by trypsin/EDTA treatment and counted on a Neubauer counting chamber. The number of cells removed from each well was within 5% to 10% of the number seeded. Figure 1 (inset) shows a calibration curve of absorbance units against cell numbers derived from three separate experiments.

Second, in two parallel experiments, the results of 3H-thymidine incorporation were compared with those from the MTT assay. Microtiter plates were set up for procoagulant assays as described in the Materials and Methods section. Half the wells were pulsed with 3H-thymidine (1 μCi/well) from 7 to 24 hours after cytokines were added. The cells in the other half of the plate were allowed to grow for 48 hours before the MTT assay was performed. The results of 3H-thymidine uptake were comparable to the cell numbers obtained from the MTT assay using the calibration curve (data not shown).

Fig 1. Effect of cytokines on the proliferation of primary (three experiments) and passaged (10 experiments) cells. Results, expressed as percentage of control cell numbers at each time point, are mean ± SEM of five experiments at day 2 and four experiments at days 4 and 7. Inset shows calibration of the MTT assay; absorbance values are mean ± SE of three experiments.

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MR 700 microplate reader (Dynatech Laboratories). The concentration of procoagulant activity was calculated from a standard curve obtained using rabbit thromboplastin (1/100,000 dilution rabbit thromboplastin = 1 U PCA).

tPA and PAI-1 assays. Confluent HUVEC (first and second passage) in 24-well plates (Costar, Cramlington, Northumbria, UK) were washed and stimulated in triplicate wells with cytokines in 1% FCS. After 24 hours, culture medium was removed and centrifuged for 3 minutes at 15,000 g to remove cell debris, and aliquots were frozen at -70°C after addition of 0.01% Tween 80. Monolayers were then washed once and treated with 0.5% Triton X, and cellular extracts were frozen at -70°C. Total human tPA (uncomplexed and inhibitor-bound) was determined using enzyme immunoassay (Biopool, Umea, Sweden). Background binding was eliminated by use of quenching antibodies. Each test sample was incubated in paired adjacent wells. To one well an excess of the coating antibody in solution was added to block binding of tPA antigen. The other well contained an irrelevant isotype-matched antibody. Hence, the difference in assay response between the two wells would be highly specific for tPA antigen. The concentration of tPA antigen in each sample was extrapolated from a standard curve obtained using single-chain recombinant human tPA (rhPA). The interassay variability was less than 10%. PAI-1 antigen (both active and "latent" forms) was assayed by enzyme immunoassay, using a similar double-antibody principle to control for immunologic specificity (Biopool). The concentration of PAI-1 in each sample was calculated from a standard curve obtained using PAI-1 standard plasma supplied by Biopool. The interassay variability was 10%.

GM-CSF receptor assays. HUVEC (third through fourth passage) were grown to confluence as described and detached using trypsin/EDTA or EGTA alone. After two washes with RPMI 1640/20% FCS, cells were resuspended in RPMI 1640/20% FCS at 4 x 10^5 cells/ml. Cells were incubated with varying concentrations of ^125-I-GM-CSF (specific activity 4.2 to 6.8 x 10^6 cpm/ng) for 4 hours at 37°C. Each sample was incubated with or without a 50-fold excess of unlabeled GM-CSF to control for nonspecific binding. Triplicate samples were layered onto chilled 20% glycerol in RPMI 1640, centrifuged at 7,000 g for 60 seconds, and snap-frozen in liquid nitrogen. The associated radioactivity of the cell pellet was measured in an automated gamma-counter (LKB, Milton Keynes, UK). An identical protocol used human peripheral neutrophils purified by dextran sedimentation of whole blood, followed by centrifugation over Lymphoprep 1077 (Nycomed, Oslo, Norway).

RESULTS

Cell proliferation. The effect of cytokines on endothelial cell proliferation was studied by culturing cells in the continuous presence of ECGS 50 μg/mL, IL-1β 5 U/mL, GM-CSF 1 ng/mL to 1 μg/mL, G-CSF 1 ng/mL to 100 ng/mL, or diluent as control in medium with 20% FCS. Cell proliferation at various time points was quantitated using an MTT assay and converting the absorbance values into actual cell numbers using the calibration curve (Fig 1, inset). The data for passaged and primary cells was combined because there was no difference between the two. Figure 1 shows that when present continuously, ECGS 50 μg/mL increased endothelial cell proliferation by 305% ± 45% (n=5, P < .01) as compared with controls at 4 days; this enhancement increased to 596% ± 137% (n=5, P < .01) after 7 days in culture. Neither GM-CSF nor G-CSF had any effect on cell growth at concentrations ranging from 1 ng/mL to 1 μg/mL. IL-1β 5 U/mL produced a small decrease in growth at 4 days to 85% ± 11% (n=5, not significant by paired t-test). In an attempt to maximize any response to added cytokines, in a second series of experiments, cultures were grown to ≤50% confluence in full culture medium and then "starved" for 48 hours in 1% FCS with 0.5% bovine serum albumin (BSA, fraction V, Sigma). Cells were then treated with cytokines in the presence of 2% FCS, and cell proliferation was assessed at various times by MTT assay. Data from primary and passaged cells were pooled. Again, ECGS enhanced proliferation of HUVEC by 342% ± 63.2% (n = 10, P < .01) over control cells at 4 days (Fig 2). There was no effect of GM-CSF (1 to 100 ng/mL), G-CSF (1 to 100 ng/mL) or IL-1β (1 to 10 U/mL) in this assay system.

Procoagulant activity. The effect of cytokines on expression of procoagulant activity (PCA) by HUVEC was studied by incubating confluent cultures with cytokines in medium with 10% FCS for varying times. Unstimulated HUVEC contained low levels of PCA (3.22 ± 0.44 PCA U per well, mean ± SD, n = 10). After 4-hour incubation with IL-1β, 10 U/mL, PCA increased to 1,721% ± 376% of control levels (n = 7, P < .005), whereas TNF 10 U/mL produced a more modest increase of 247% ± 71% of control levels (n = 4) (Fig 3). γ-IFN (50 U/mL) and GM-CSF (1 to 100 ng/mL) had no direct effect on PCA. γ-IFN, however, was able to augment the PCA production by endothelial cells in response to IL-1β. Figure 4, representative of two experiments, shows the effect of a 16-hour preincubation with γ-IFN (50 U/mL) before addition of IL-1 (1 to 10 U/mL) for 4 more hours. GM-CSF (1 to 100 ng/mL) had no effect in three experiments. In two other experiments, GM-CSF was added simultaneously with IL-1β, and incubation proceeded for 4 to 24 hours. No modulation of IL-1β-induced PCA expression was observed.

tPA and PAI-1 expression. The influence of cytokines on the fibrinolytic properties of HUVEC was studied by 24-hour stimulation of confluent monolayers of cells with addition of 1% FCS. This concentration of serum was
chosen because it has been reported to result in near optimum tPA expression by primary cultured endothelial cells. The unstimulated level of tPA antigen secreted by HUVEC is low (2.4 ± 0.2 ng/10^6 cells, n = 5) and increases to 314% ± 72% of control levels (n = 5, P < .025) after 24-hour incubation with thrombin 4 U/mL (Fig 5). IL-1β (10 U/mL) produced a small but insignificant decrease to 85% ± 9% of control levels (n = 3), whereas GM-CSF (1 to 100 ng/mL) and G-CSF (1 to 100 ng/mL) had no effect on tPA expression. Cell-associated tPA antigen levels were below the level of detection of the assay.

Secreted PAI-1 antigen expression by HUVEC was increased to 275% ± 44% and 180% ± 12% of controls (n = 5, P < .0005 for both) after 24-h incubation with IL-1β 10 U/mL and thrombin 4 U/mL, respectively (Fig 6). Similarly, cell-associated PAI-1 antigen was increased to 266% ± 108% (n = 3, NS by Student’s t test) and 195% ± 54% (n = 3, NS) of control levels after 24-hour incubation with IL-1 and thrombin, respectively. GM-CSF and G-CSF had no effect on secreted or cellular PAI-1.

**GM-CSF receptor assay.** In view of the lack of effect of GM-CSF on HUVEC in our system, we wished to determine the number of GM-CSF receptors present on these cells. HUVEC at third and fourth passage, pooled from at least three umbilical cords, were detached by treatment with trypsin/EDTA and used in a binding assay with 125I-GM-CSF as detailed in the Materials and Methods section. In two experiments, no specific binding of 125I-GM-CSF was noted (data not shown). In case trypsin treatment might have cleaved off any GM-CSF receptors on the cells, two other experiments were performed using HUVEC grown on gelatin (0.05%)-coated flasks, and removed by treatment with 1 mmol/L EGTA (10 minutes at 37°C). Again, no specific binding of 125I-GM-CSF to endothelial cells was observed (Fig 7A), whereas peripheral blood (PB) neutrophils exhibited significant specific binding. Figure 7B is representative of three experiments. Scatchard analysis showed the presence of 80 ± 78 (mean ± SEM) high-affinity receptors per neutrophil, with an affinity constant of 57 ± 15 pmol/L. With 3 × 10^6 cells per data point, 10 receptors per cell can be calculated to give specific binding of 125I-GM-CSF 50% above the background binding to neutrophils. Endothelial cells exhibit higher levels of non-
specific binding of $^{125}$I-GM-CSF than neutrophils, but 25 receptors per endothelial cell should give binding values 50% above background.

The endothelial line EAhy926$^{39}$ has also been reported to express GM-CSF receptors and to respond to this cytokine.$^{5}$ We therefore repeated our assay on cells of this line, detached from plastic tissue culture flasks by EGTA treatment. These cells did not demonstrate any specific binding of $^{125}$I-GM-CSF in two experiments (data not shown).

DISCUSSION

The HUVEC used in these studies display a range of responses to cytokines. Proliferation is enhanced in response to ECGS, but not to IL-1β. IL-1β and TNF directly enhance cell-associated PCA whereas γ-IFN, although not having any direct effect on its own, primes the IL-1β response. This confirms previous reports.$^{10,11}$ Secretion of tPA by HUVECs is enhanced by thrombin, but not by IL-1β and TNF. All three agonists, however, increase both secreted and cellular PAI-1 antigen. These observations are in keeping with those of other investigators.$^{12-15}$ In these responses, the HUVEC in our culture system behave in a manner similar to that reported for other cultured human endothelial cells.

We have observed a lack of response to GM-CSF and G-CSF, however, in all the parameters described above. The failure of cultured HUVEC to proliferate in response to GM-CSF and G-CSF in our studies contrasts with the reports by Bussolino et al.$^{16,17}$ on multiply passaged endothelial cells. In addition, culturing cells up to the eighth passage in the continuous presence of GM-CSF (10 to 100 ng/mL) did not induce any responsiveness to the growth factor (data not shown). The lack of effect of GM-CSF on HUVEC cell-associated PCA has been noted by other investigators.$^{11}$

rhG-CSF has been reported to increase plasminogen activator activity in bovine aortic endothelial cells.$^{6}$ We noted no effect on TPA or PAI-1 antigen levels in primary human endothelial cells at similar doses of the same recombinant product. The functional assays for plasminogen activator activity used by Kojima et al.$^{6}$ do not distinguish between urokinase-type plasminogen activators and tPA, however, whereas our assays are specific for tPA. Furthermore, the type of plasminogen activator produced can vary with the origin of the endothelial cells as well as the conditions of stimulation.$^{36,39,40}$

Finally, in keeping with the lack of response to GM-CSF, we failed to demonstrate any GM-CSF receptors on either primary human endothelial cells or the endothelial cell line EAhy 926, although in the same experiments, neutrophils expressed $802 \pm 78$ high-affinity receptors. This again contrasts with reports by Bussolino et al, who observed $423 \pm 187$ high-affinity receptors on human endothelial cells.$^{11}$ The reason for this discrepancy is not clear, but it suggests that expression of GM-CSF receptors by cultured endothelial cells and the ability of these cells to respond to these growth factors is critically dependent on culture conditions. One should not assume that GM-CSF influences endothelial cell function in vivo.

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