Communication

Stimulation of the Na+/H+ Exchanger in Human Endothelial Cells Activated by Granulocyte- and Granulocyte-Macrophage-Colony-stimulating Factor

EVIDENCE FOR A ROLE IN PROLIFERATION AND MIGRATION*

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It has been shown that human endothelial cells (HEC) are stimulated to migrate and proliferate by granulocyte (G)- and granulocyte-macrophage (GM)-colony-stimulating factor (CSF) (Bussolino, F., Wang, J. M., Defilipii, P. Turrini, F., Sanavio, F., Edgell, C.-J. S., Aglietta, M., Arese, P., and Mantovani, A. (1989) *Nature* 337, 471–473). The rapid intracellular events initiated by these cytokines on binding to their receptors on HEC are not defined. Addition of G- or GM-CSF to HEC produced a rapid activation of Na+/H+ exchanger resulting in an increase in intracellular pH (pH$_i$). Both cytokines induced an alkaline displacement in the pH$_i$ dependence of the exchanger without affecting the affinity for external Na+ (Na$_o$) and the rate of exchanger. Ethylisopropylamiloride, a selective inhibitor of the Na+/H+ exchanger, inhibited the intracellular alkalinization, the migration, and proliferation induced by G- and GM-CSF. The data indicate that G- and GM-CSF initiate a rapid exchange of Na+ and H+ by means of the Na+/H+ exchanger and that this ethylisopropylamiloride-sensitive ions flux is important to the biological effects of these cytokines on HEC.

G-[1] and GM-CSF are growth factor glycoproteins that control the maturation process of granulocyte-macrophage progenitors and affect the functions of mature cells (1, 2).

These factors have been considered restricted to the hematopoietic system. It was observed recently that G- and GM-CSF activate nonhematopoietic cells, namely endothelial cells (3, 4), keratinocytes (5), and some malignant cells (6–8). G- and GM-CSF induce migration and proliferation of human endothelial cells (HEC) (3), and GM-CSF causes the growth of keratinocytes (5) and of several tumoral cell lines (6, 7). HEC and tumor cells have specific receptors for G- and GM-CSF similar in number and affinity to those present on myelomonocytic cells (3, 6, 8).

The pathways involved in G- and GM-CSF signal transduction are not known in nonhematopoietic cells. Even in myelomonocytic cells relatively little, and to some extent conflicting, information is available on signal transduction pathways utilized by these cytokines. In mature granulocytes, G- and GM-CSF do not change the intracellular concentration of inositol phosphates and Ca^{2+} nor activate protein kinase C (9–11), but GM-CSF causes an increase in Na+ influx and an increase in pH$_i$ coupled to a rapid increase in tyrosine phosphorylation levels (12). In addition, the commonly observed increases in pH$_i$ in neutrophils stimulated with chemotractants are inhibited in neutrophils "primed" by GM-CSF (10, 12). This may indicate that the activation of the Na+/H+ exchange mechanism by GM-CSF inhibits further stimulation of this system with chemotractants, thus explaining the observed increased acidification in GM-CSF-primed neutrophils stimulated with chemotactic peptides (10, 13). In contrast to its effect on mature nonproliferating cells, GM-CSF directly stimulates membrane-associated protein kinase C activity and inositol phosphates generation in the HL-60 promyelocytic cell line (14) and activates Na+/H+ antiport in the AML-193 monocytic cell line.[2]

The Na+/H+ exchanger is an electroneutral membrane system that exchanges intracellular H+ for extracellular Na+ (15, 16). It is involved in the regulation of intracellular pH (pH$_i$), in the control of cellular volume (15, 16), and it is activated by growth factors (17–19) and circulating mediators or hormones (20–22). The present study demonstrates that the Na+/H+ antiport is stimulated by G- and GM-CSF in HEC and that it plays a direct role in G- and GM-CSF-mediated proliferation and migration of HEC.

EXPERIMENTAL PROCEDURES

Cell Cultures—HEC isolated from human umbilical cord vein were grown in M199 and characterized as previously described (3, 23). All cultures, used at III–V passage, were checked for leukocyte contamination using monoclonal antibodies anti-CD11b, -Mo2, and -T3 and FACS with negative results.

Fluorimetric pH$_i$ Monitoring—pH$_i$ and Na+-dependent H+ efflux in HEC grown on glass coverslips coated with fibronectin (10 μg/ml) (Sigma) were assessed essentially as previously described (23). HEC were incubated for 15 min at 37 °C with 30 μM 6-carboxyfluorescein diacetate (Calbiochem) in Na-Hepes buffer (145 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 10 mM Hepes, 1 mM CaCl₂, 10 mM glucose, pH 7.4). After washings, the coverslip was inserted into a thermostatted cuvette containing 1 ml of Na-Hepes buffer in a Perkin-Elmer LS-5 spectrofluorimeter, and the pH$_i$-dependent emission was continuously recorded at 37 °C (excitation, 490 and 430 nm; emission, 520 nm; slit width, 5 nm). The ratio of emitted fluorescence signals (520 nm) permits calculation of pH$_i$ that is independent on cell number, dye loading, and dye leakage. The fluorescence signal was calibrated as a

[1] The abbreviations used are: G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte-macrophage-colony-stimulating factor; CSF, colony-stimulating factor; FCS, fetal calf serum; pH$_i$, intracellular pH; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Na$_o$, extracellular Na+; EIPA, ethylisopropylamiloride; HEC, human endothelial cells; M199, medium 199 containing 20 mM NaHCO₃.

[2] D. Caracciolo and A. Bosia, manuscript in preparation.

function of pH$_i$ by using Hepes buffer containing 150 mM KCl instead of NaCl (pH 6.2–7.4) (K-Hepes buffer) and 4 μM nigericin (Sigma) (24). The ratio of the fluorescence signals at 490 nm to that at 430 nm as a function of pH$_i$ followed a standard pH titration curve, and a nearly linear relationship between pH and fluorescence over the pH range 6.2–7.4 was observed. To measure the pH$_i$ dependence of H$^+$ efflux, the rates of H$^+$ efflux were measured after acidification of the cells to different pH$_i$ (from 6.2 to 7.2 in K-Hepes buffer) in the presence of nigericin. After blocking the effect of nigericin by the addition of albumin (5 mg/ml) (25) and removing K-Hepes buffer, the fluorescence signal was recorded in a modified Na-Hepes buffer (50 mM NaCl and 90 mM N-methyl-D-glucamine chloride instead of 145 mM NaCl). Na$_o$ dependence of the rates of H$^+$ efflux in acid-loaded cells (pH 6.2–6.3) was measured by monitoring fluorescence signals in Hepes-isotonic buffer containing different NaCl concentrations (0–100 mM) (N-methyl-D-glucamine chloride was used as an osmotic balance), pH 7.4.

The cytoplasmic buffering capacity was determined by subjecting HEC to NH$_4$Cl as previously detailed (23) and calculated as [NH$_4^+$]/pH$_i$ (26) using a pK_a value for ammonium of 9.21. The rates of H$^+$ efflux were calculated as the product of the rate of pH$_i$ change (in pH units/min) times the buffering power (mmol/liter/pH unit). The rates of pH$_i$ change were measured directly from the fluorescence recordings.

Proliferation Assay—2 × 10^4 HEC were plated in 24-well plates (Costar Corp., Cambridge, MA) coated with gelatin (Difco) (0.05%, 1 h, 22 °C), and grown for 24 h as described above. Medium was then removed and replaced with M199 containing 10% FCS with or without human recombinant G-CSF (a gift of Dr. L. Souza, Amgen, Thousand Oaks, CA; specific activity, 1.5 × 10^8 units/mg) (200 pM), human recombinant GM-CSF (a gift of Dr. D. Krumwieh, Behringwerke, Marburg, Federal Republic of Germany) (200 pM), EIPA (Merck) (5 μM), or G-CSF/GM-CSF + EIPA . The medium containing these substances was replaced every 48 h. HEC number was estimated by fluorimetric DNA assay by using the DNA intercalating fluorescent compound bisbenzimide (27). A calibration curve was set up with known numbers of suspended HEC. Proportionality between fluorescence and cell counts was observed up to 1 × 10^6 HEC. G- and GM-CSF were endotoxin-free as assessed by *Limulus* assay (Sigma). Cell viability was assessed by trypan blue exclusion (>94% in all conditions tested).

Chemotaxis Assay—Chemotaxis assay were performed with a 48-well modified Boyden chamber technique (3, 28). Polycarbonate filters (5-μm pore size, polyvinylpyrrolidone-free, Nucleopore (Corp., Pleasanton, CA) were soaked in 0.5 M acetic acid, washed with phosphate-buffered saline (pH 7.4), incubated for 24 h in 0.01% gelatin (Difco), and air dried. 200 pM G- or GM-CSF in M199 containing 1% FCS were seeded in the lower compartments of the chamber. HEC (2 × 10^6/ml of M199 containing 1% FCS) were preincubated for 10 min with different concentrations of EIPA at room temperature. 0.05 ml of cell suspension in M199 containing 1% FCS were then seeded in the upper compartments. After 6 h of incubation at 37 °C (5% CO$_2$) the upper surface of the filter was scraped with a rubber policeman. The filters were fixed and stained with Diff-Quick (Harleco, Gibbstown, NJ), and 10 oil immersion fields were counted after coding samples.

RESULTS

Basal pH$_i$ of HEC grown on coverslips in Na-Hepes buffer at 37 °C was 7.35 ± 0.02 ($n = 15$). Addition of G- or GM-CSF at a biologically active concentration (200 pM) (3) caused a sustained alkalinization (range, 0.1–0.25 pH units) reaching its maximum after 5 min (Fig. 1). Boiled and biologically inactive (3) G- and GM-CSF were ineffective. If HEC were placed in Na-Hepes buffer containing 50 μM EIPA, a potent and specific inhibitor of Na$^+$/H$^+$ exchange (29, 30), for 5 min at 37 °C G- and GM-CSF-induced alkalinization was abolished, supporting the involvement of Na$^+$/H$^+$ exchange. This supposition was confirmed in experiments performed in Na$^+$-free Hepes buffer (NaCl isotonically replaced by 145 mM N-methyl-D-glucamine chloride), in which no alkalinization was observed (Fig. 1).

To study the [Na]$_o$ dependence of the exchanger, HEC initially acidified with nigericin to pH$_i$ 6.2–6.3 were placed in
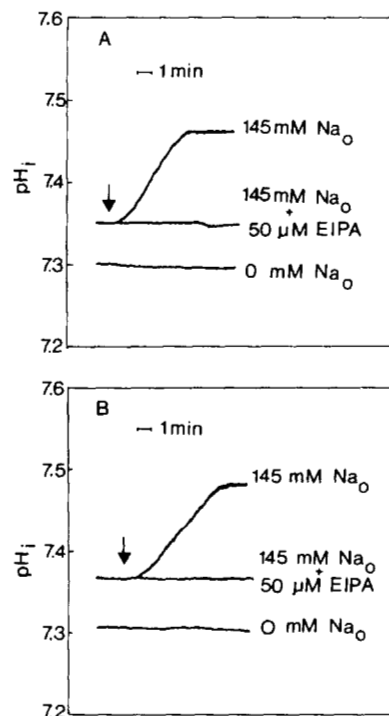

FIG. 1. **Effect of G- (*A*) and GM-CSF (*B*) on pH$_i$ of HEC.** 6-Carboxyfluorescein diacetate-loaded HEC grown on coverslip were incubated in Na-Hepes buffer at 37 °C in a fluorimeter cuvette. *Arrows* indicate the addition of G- or GM-CSF (200 pM). Alternatively, experiments were done with HEC preincubated with 50 μM EIPA for 10 min at 37 °C or Na-free Hepes buffer in which Na$^+$ was isoosmotically replaced by N-methyl-D-glucamine$^+$. Traces are representative of six similar experiments.

Hepes buffer containing different Na$^+$ concentrations isoosmotically balanced with N-methyl-D-glucamine$^+$. Fig. 2A shows the kinetic properties of the Na$^+$/H$^+$ exchanger for Na$_o$ in control and in G- and GM-CSF-treated cells. Under our experimental conditions, control cells exchanged Na$^+$ with a $K_{m(Na)} = 26.5 \pm 4.3$ and $V_{max} = 43.2 \pm 5.1$ mmol of H$^+$/liter/min ($n = 4$). In HEC treated with 200 pM G- or GM-CSF for 10 min prior to exposure to different [Na]$_o$, Na$^+$ was exchanged with $K_{m(Na)} = 19.3 \pm 5.1$ and $V_{max} = 46.3 \pm 2.1$ mmol of H$^+$/liter/min (G-CSF) and with $K_{m(Na)} = 18.3 \pm 2.5$ and $V_{max} = 45.8 \pm 6.9$ mmol of H$^+$/liter/min (GM-CSF), respectively ($n = 4$). The affinity of the exchanger for Na$_o$ was slightly increased by both agonists; however, these differences were not statistically significant. To determine whether G- and GM-CSF modified the Na$^+$/H$^+$ exchanger affinity for intracellular H$^+$, pH$_i$ was preset by nigericin technique to values ranging from 6.2 to 7.4. The rate of alkalinization in Hepes buffer containing 50 mM NaCl was then measured in HEC preincubated for 10 min at 37 °C with 200 pM G- or GM-CSF. A typical experiment is illustrated in Fig. 2B. In both unstimulated and stimulated HEC, decreasing pH$_i$ increased the activity of the Na$^+$/H$^+$ exchanger. However, in control cells, the rate of H$^+$ efflux was almost negligible at pH$_i \leq 7.2$ but increased strongly as the cytoplasm was acidified. In G- and GM-CSF-treated cells, Na$^+$-induced H$^+$ efflux was still observed at pH$_i$ 7.2 and became negligible only at pH$_i \geq 7.3$, that is consistent with the final pH$_i$ attained after addition of the two agonists in Na-Hepes buffer (Fig. 1). In three similar experiments in triplicate, the Na$^+$/H$^+$ exchanger was inactive at pH$_i$: 7.05 ± 0.16 in control cells and 7.37 ± 0.07 ($p < 0.05$) and 7.36 ± 0.05 ($p < 0.02$) in G- and GM-CSF-stimulated HEC, respectively.
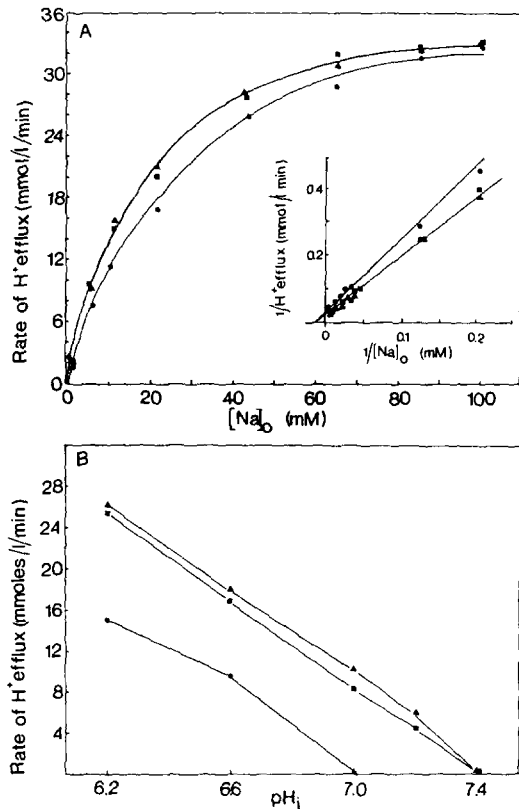
FIG. 2. The Na_o (A) and pH_i (B) dependence of the Na^+/H^+ exchanger in unstimulated (●) and G- (■) or GM-CSF (▲)-stimulated HEC. 6-Carboxyfluorescein diacetate-loaded HEC were stimulated with 200 pM G- or GM-CSF or solvent in Na-Hepes buffer for 10 min at 37 °C and then placed in a thermostatted fluorimetric cuvette. A, HEC were acid-loaded (pH_i 6.25) with nigericin as described under "Experimental Procedures" and then placed in Hepes buffer containing different NaCl concentrations (isoosmolarity was maintained by the addition of N-methyl-D-glucamine chloride). The rate of H^+ efflux was calculated as described under "Experimental Procedures." Inset, data presented as a double-reciprocal plot (Lineweaver-Burk) calculated by the ENZFITTER program (IBM). B, HEC were acid-loaded with nigericin to the pH_i indicated on the abscissa and then placed in a modified Hepes buffer containing 50 mM NaCl and 90 mM D-methyl-N-glucamine chloride. Each point is the mean of three determinations in one typical experiment. Three experiments were done with similar results.

To evaluate the relationship between G- and GM-CSF-induced activation of Na^+/H^+ antiport and their property to activate migration and proliferation of HEC, we performed experiments with EIPA, a selective and potent inhibitor of Na^+/H^+ exchange (29, 30). HEC cultured in HCO_3^--containing medium in the presence of 200 pM G- or GM-CSF increased progressively in number over the 7 days of observation. By contrast, HEC kept for 7 days in the presence of 5 μM EIPA and G- or GM-CSF showed a significant inhibition of growth (−45%) (Fig. 3A). EIPA inhibited also G- and GM-CSF-induced HEC migration in a dose-dependent manner (Fig. 3B). 100 μM EIPA completely blocked the migration (not shown), whereas 10 μM induced 41 and 52% inhibition of G- and GM-CSF-induced migration, respectively.

To exclude a possible toxicity of EIPA, HEC were treated for 6 h with 50 μM EIPA in M199 containing 20% FCS and then extensively washed and tested for proliferative capability in the presence of basic fibroblast growth factor (10 ng/ml, Amersham, Bucks, United Kingdom), heparin (100 μg/ml), and 20% FCS. After 4 days the number of cells in control and EIPA-treated cells showed a 1.3–2-fold increase. Furthermore,
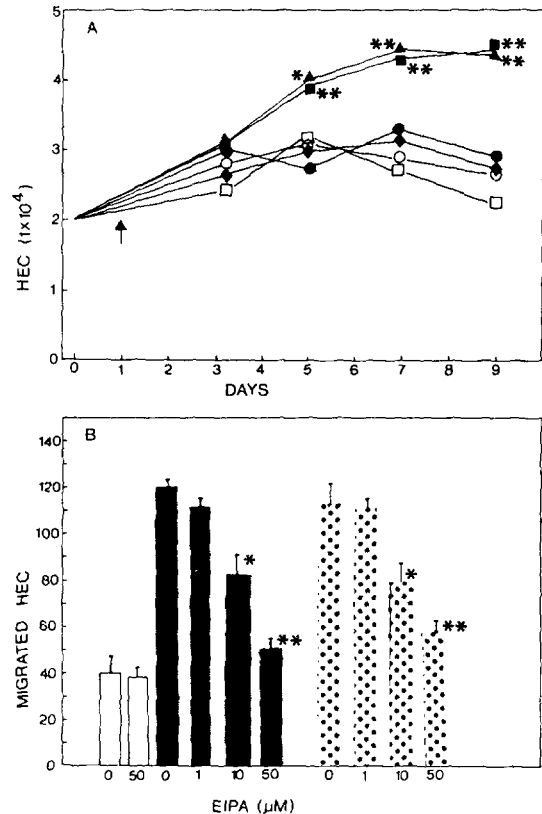

FIG. 3. Effect of EIPA on proliferation (A) and migration (B) of HEC induced by G- or GM-CSF (200 pM). A, 2×10^4 HEC were plated in a 24-well plate coated with gelatin as described under "Experimental Procedures." After 24 h medium was replaced with M199 supplemented with 10% FCS in the presence or absence of G- or GM-CSF and/or 5 μM EIPA. The medium containing these substances was replaced every 48 h. Shown is the mean of four experiments done in quadruplicate. The S.D. ranged from ±1036 to ±7120 and was omitted to simplify the picture. O, control; ■, G-CSF; ▲, GM-CSF; □, EIPA; ●, G-CSF + EIPA; ◆, GM-CSF + EIPA. B, G- and GM-CSF in M199 containing 1% FCS were placed in the lower compartments of the chemotaxis chamber. HEC (2×10^6/ml) were preincubated with EIPA at room temperature for 10 min, and 0.05 ml of cell suspension were seeded in the upper compartments. Results are presented as the number of migrated HEC (mean ± S.D. of three experiments done in triplicate) in 10 oil immersion fields. □, control; ■, G-CSF; ▨, GM-CSF; *, $p < 0.05$; **, $p < 0.01$ (Dunnet's test).

EIPA in all conditions tested did not induce more than 3–7% cell death evaluated by trypan blue exclusion.

DISCUSSION

We have shown earlier that an amiloride-inhibitable Na^+/H^+ exchanger is present in the membrane of HEC and that its activation is involved in the regulation of Ca^{2+} influx and platelet-activating factor synthesis induced by thrombin (23). We extend here this observation, showing that G- and GM-CSF, which are able to promote migration and proliferation of HEC by a specific receptor mechanism (3), activate this exchanger in HEC. Furthermore, EIPA, one of the most potent inhibitors of this electroneutral transporter (29, 30), inhibits Na^+-induced H^+ efflux, migration, and proliferation, suggesting that Na^+/H^+ exchange plays an important role in the activation of these HEC responses. Na^+/H^+ exchange is accelerated in response to the biologically active concentration of G- and GM-CSF, which elicit a readily detectable cytoplasmic alkalinization of 0.1–0.25 pH units.

The mechanism of G- and GM-CSF-induced activation of

the antiport is apparently similar to that described in several systems (31–33). Stimulation involves an alkaline shift in the pH$_i$ dependence of Na$^+$/H$^+$ exchange, with little change in other kinetic parameters. This shift apparently reflects an altered behavior of the cytoplasmic modifier site (34), as this site largely determines the pH$_i$ sensitivity of the exchanger. The set point of the modifier appears to be adjusted upward by 0.1–0.2 pH units, which is the magnitude of the alkalosis recorded in the Na$^+$-containing medium after G- or GM-CSF challenge (Fig. 1).

We investigated whether a pharmacological inhibition of Na$^+$/H$^+$ exchanger could affect the G- and GM-CSF-induced HEC activation by using EIPA. This is a highly potent derivative of amiloride that inhibits the Na$^+$/H$^+$ exchanger in various cells, with a considerable selectivity in the concentration range used in the present study (29, 30). Our experiments show that G- and GM-CSF-induced migration and proliferation of HEC in bicarbonate-containing medium are strongly inhibited by EIPA. In proliferation experiments, the presence of bicarbonate rules out the possibility that inhibition of the growth might result from an alteration of pH homeostasis, as could be the case when both Na$^+$/H$^+$ exchange and HCO$_3$-dependent processes are impaired (35, 36).

The results presented here show that G- and GM-CSF activate Na$^+$/H$^+$ exchanger in HEC by affecting the affinity for intracellular H$^+$. The finding that the selective inhibitor EIPA blocks the migration and proliferation induced by these cytokines in HEC is consistent with the hypothesis that altered activity of tha Na$^+$/H$^+$ exchanger plays a role in determining the ultimate biological responses of these cells. The role of Na$^+$/H$^+$ exchanger as a signal transduction mechanism has recently been questioned (37–40). Activation of Na$^+$/H$^+$ exchanger in CSF-treated HEC may represent a mechanism of enhancement of the housekeeping ability of these cells in anticipation of increased metabolic production of H$^+$ associated with migration and proliferation.

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