HERG K$^{+}$ Channels Activation during $\beta_1$ Integrin-mediated Adhesion to Fibronectin Induces an Up-regulation of $\alpha_v\beta_3$ Integrin in the Preosteoclastic Leukemia Cell Line FLG 29.1*

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Integrin receptors have been demonstrated to mediate either “inside-to-out” and “outside-to-in” signals, and by this way are capable of regulating many cellular functions, such as cell growth and differentiation, cell migration, and activation. Among the various integrin-centered signaling pathways discovered so far, we demonstrated that the modulation of the electrical potential of the plasma membrane (V$\text{REST}$) is an early integrin-mediated signal, which is related to neurite emission in neuroblastoma cells. This modulation is sustained by the activation of HERG K$^{+}$ channels, encoded by the ether-a`go-go-related gene (herg). The involvement of integrin-mediated signaling is being discovered in the hematopoietic system; in particular, osteoclasts are generated as well as induced to differentiate by interaction of osteoclast progenitors with the stromal cells, through the involvement of integrin receptors. We studied the effects of cell interaction with the extracellular matrix protein fibronectin (FN) in a human leukemic preosteoclastic cell line (FLG 29.1 cells), which has been demonstrated to express HERG currents. We report here that FLG 29.1 cells indeed adhere to purified FN through integrin receptors, and that this adhesion induces an osteoclast phenotype in these cells, as evidenced by the appearance of tartrate-resistant acid phosphatase, as well as by the increased expression of CD51/$\alpha_v\beta_3$ integrin and calcitonin receptor. An early activation of HERG current (I$\text{HERG}$), without any increase in herg RNA or modifications of HERG protein was also observed in FN-adhering cells. This activation is apparently sustained by the $\beta_1$ integrin subunit activation, through the involvement of a pertussis-toxin sensitive G$\text{i}$ protein, and appears to be a determinant signal for the up-regulation of $\alpha_v\beta_3$ integrin, as well as for the increased expression of calcitonin receptor.

The integrin family of adhesion receptors functions not only as ligands for the extracellular matrix (ECM), but can influence many aspects of cell behavior, including morphology, adhesion, migration, as well as cellular proliferation and differentiation. In fulfilling these functions, integrins are not simply adhesion receptors, but can affect many signaling pathways, and therefore impinge upon complex cellular activities. Therefore integrin function itself is highly regulated, largely through the formation of specific associations with both structural and regulatory components within the cell. Recently, much research has focused on elucidating the molecular mechanisms, which control integrin function and transduce integrin-mediated signaling events.

In particular, in the hemopoietic system, it is well known that adhesive interactions between hemopoietic precursors and the bone marrow microenvironment play a critical role in regulating hemopoiesis (1, 2), and that such interactions are often mediated by integrin receptors (3, 4). Among hemopoietic precursors, the progenitors of osteoclasts are formed through a contact-dependent interaction between bipotential osteoclast-macrophage precursors and stromal cells, which express osteoclast forming activity (5, 6). Furthermore, osteoclast differentiation and activation leading to bone remodeling is finely regulated by interaction of osteoclasts with stromal cells (reviewed in Ref. 7) through the involvement of integrin receptors, which are numerous on the osteoclast plasma membrane (8). Therefore, osteoclast precursors can be envisaged as a good model wherein studying the relationships between the ECM, integrin-mediated signals, and the induction of cell differentiation/activation.

Among the various integrin-centered signaling pathways discovered so far (9, 10), we demonstrated that the modulation of V$\text{REST}$ is an early integrin-mediated signal, which is related to neurite emission in neuroblastoma cells (11–13). This modulation is sustained by the activation of a peculiar type of K$^{+}$ channels, the HERG channels (14, 15), encoded by the ether-a`-go-go-related gene (herg), human-eg-related gene; HERG, herg-encoded protein; I$\text{HERG}$, HERG current; FN, fibronectin; VN, vitronectin; V$\text{REST}$, membrane resting potential; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; TRAP, tartrate-resistant acid phosphatase; RTPCR, reverse transcriptase polymerase chain reaction; CIR, calcitonin receptor; mAb, monoclonal antibody; bp, base pair(s); PKC, protein kinase C; PTX, pertussis toxin; gapdh, glyceraldehyde-3-phosphate dehydrogenase.

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The abbreviations used are: ECM, extracellular matrix; herg, human-eg-related gene; HERG, herg-encoded protein; I$\text{HERG}$, HERG current; FN, fibronectin; VN, vitronectin; V$\text{REST}$, membrane resting potential; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; TRAP, tartrate-resistant acid phosphatase; RTPCR, reverse transcriptase polymerase chain reaction; CIR, calcitonin receptor; mAb, monoclonal antibody; bp, base pair(s); PKC, protein kinase C; PTX, pertussis toxin; gapdh, glyceraldehyde-3-phosphate dehydrogenase.
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go-go-related gene (herg). These are channels with limited hyperpolarizing potency, clamping \( V_{\text{rest}} \) to substantially depolarized values (around −30 mV), and are expressed in the heart (16–18), embryonic neuroblasts (19), as well as in tumor cells of various histogenesis (12). We found that a human leukemia cell line, FLG 29.1 cell, expressed herg, and an HERG current (I_{HERG}) with a very fast deactivation kinetics, which apparently justifies the low, depolarized value of their \( V_{\text{rest}} \) (12, 20). These cells derive from a patient with an M5a-type leukemia (21) and represent immature preosteoclastic precursors, as revealed by their treatment with phorbol esters (22). They are capable of adhering to bone endothelium, possibly through fibronectin (FN) molecules produced by the endothelial cells (23), and this interaction could influence the maturation of these cells through the osteoclastic pathway. We therefore used the preosteoclastic FLG 29.1 cells to study HERG K⁺ channel-centered signals, elicited by integrin-mediated adhesion to the ECM molecule FN, as a model for hemopoietic precursors, and their relationships with the process of osteoclastic differentiation.

We report here that FLG 29.1 cells indeed adhere to purified FN through integrin receptors, and that this adhesion is accompanied by the appearance of osteoclast differentiation markers, namely tartrate-resistant acid phosphatase (TRAP), calcitonin receptor (CtR), as well as CD51/\(\alpha_2\) integrin. FN also induces the activation of I_{HERG}, without any increase in herg RNA or modifications of HERG protein. This activation is apparently sustained by the \(\beta_1\) integrin subunit activation, through the involvement of a pertussis toxin-sensitive G\(_i\) protein, and appears to be a determinant signal for the up-regulation of \(\alpha_2\) integrin, as well as for the increased expression of CtR.

**Experimental Procedures**

**Cell Culture—**FLG 29.1 were obtained in Dr. P. A. Bernabei’s laboratory (Hematological Unit, Florence, Italy) as previously reported (22). Cells were routinely cultured in RPMI medium (Hyclone) supplemented with 10% fetal calf serum (Hyclone) (complete medium) and incubated at 37 °C in 10% CO₂. For experiments, cells were harvested from freshly seeded preparatory cultures and resuspended (2.5 × 10⁵ cells/ml) in RPMI containing 250 μg/ml heat-inactivated bovine serum albumin (BSA) (RPMI + BSA) (for cell adhesion experiments) or in complete medium (for differentiation experiments). Cells were then plated onto either 35-mm Petri dishes (Costar) (for patch clamp experiments) or dishes in which glass slides had been accommodated (for immunocytochemistry experiments) or 10-cm Petri dishes (for RNA or protein extraction).<br>

**Preparation of Substrates and Coating of Culture Dishes—**Heat-inactivated BSA was prepared as described previously (11). Coating of culture dishes with FN or vitronectin (VN) (for cell adhesion experiments) or in complete medium (for differentiation experiments) was performed by adding two proteins, prepared as described in Ref. 11, at 100 μg/ml in RPMI at 37 °C for 1 h. Thereafter, FN or VN solution was poured off and dishes were further incubated for 30 min with RPMI + BSA to saturate the free binding sites of the culture dish surface. Dishes were then immediately used for experiments or stored with PBS at 4 °C and used afterward (1 or 2 days).

Experiments with substrate-bound as well as soluble anti-integrin antibodies were performed by using the following antibodies: (a) anti-\(\beta_1\) monoclonal antibodies: mAb BV7 (24) and mAb TS2/16; (b) anti-\(\beta_2\) monoclonal antibody (mAb B212) (25); (c) anti-\(\alpha_5\) monoclonal antibody (P2) (26), and following the procedure essentially according to Arcangeli et al. (13). Briefly, the antibodies were diluted at 20 μg/ml in RPMI, and added to Petri dishes at 37 °C for 1 h. After coating the plastic surface, dishes were further incubated for 30 min with RPMI + BSA as above, and thereafter immediately used for patch clamp experiments (see below). For experiments with soluble anti-\(\beta_2\) antibody (mAb TS2/16), the antibody was diluted at 20 μg/ml in RPMI medium + BSA, and cells were added to the solution at 2.5 × 10⁵/ml for 10 min at room temperature, then transferred to BSA-coated Petri dishes, and incubated for further 15 min at 37 °C.

**Adhesion Assay—**Adhesion assay was performed essentially according to Arcangeli et al. (11). Briefly, stock cultures were radiolabeled during 36 h of exponential growth in RPMI medium containing 1 μCi/ml [methyl-\(^3\)H]thymidine (specific activity 24 Ci/mmol). After this time, cells were harvested, pelleted, and resuspended in RPMI + BSA. Aliquots of cells (14 × 10^6) were inoculated into each well of 96-well clusters (Corning-Costar), previously coated with the adhesive proteins (see above). In the appropriate samples, blocking antibodies to FN-receptor (αFN-R; 1.50 dilution) (11), \(\beta_1\) subunit (mAb B212, 9 μg/ml), or \(\beta_2\) subunit (mAb TS2/16, 8 μg/ml) were added to Petri dishes at 37 °C for 5 min prior to the mAb B212 was used; cells were preincubated with the antibody for 20 min at 4 °C, as described by Defilippi et al. (25). When needed, GRGDSP (Telios) or GRGESP (Telios) were added just before cell seeding at 0.5 μg/ml (final concentration). After 60 min of incubation, the medium was aspirated off and adherent cells gently rinsed twice with PBS containing 1% FBS, and were then eluted with 50 μl of 1% SDS in 0.1 M NaOH for 1 h. Radioactive solubilized cells were quantified by scintillation counting and compared on a percent basis with the radioactivities of the FN-seeded cells.

**Induction of Cell Differentiation—**For induction of cell differentiation, TPA (Sigma) was dissolved in dimethyl sulfoxide at 10⁻⁶ M and added to complete medium at a final concentration of 10⁻⁸ M.

**Pertussis Toxin Treatment—**Pertussis toxin (Calbiochem) dissolved in water was added to cell cultures at a final concentration of 100 ng/ml, and cells were incubated in its presence for 14–20 h. After this time, cells were harvested and resuspended in RPMI + BSA in the absence of the toxin, and seeded on BSA-, FN-, or anti-\(\beta_1\) antibody (mAb TS2/16)-coated dishes for 1 h.

**Patch Clamp Recordings—**Cells plated on dishes were incubated at 37 °C for various times. Patch clamp experiments were performed at room temperature with an amplifier Axopatch 1-D (Axon Instruments, Foster City, CA), replacing the Petri dishes every 30 min. The whole cell configuration of the patch clamp technique (27) was employed using pipettes (borosilicate glass; Hilgenberg, Germany) whose resistance was in the range 5–5 MΩ. Extracellular solutions were delivered through a 9-hole (0.6 mm), remote-controlled linear positioner placed near the cell under study. The standard extracellular solution contained (mM): NaCl 130, KCl 5, CaCl₂ 2, MgCl₂ 2, Hepes-NaOH 10, glucose 5, pH 7.4. The standard pipette solution at [Ca²⁺] = 10⁻⁷ M contained (mM): K⁺ aspartate 130, NaCl 10, MgCl₂ 2, CaCl₂ 4, EGTA 10, Hepes KOH 10, pH 7.4. Gigaseal resistance were in the range 1–10 GΩ. Whole cell currents were filtered at 2 KHz. For precise measurement of the gating parameters of the inward rectifier channels, we carefully compensated pipette and cell capacitance and the series resistance before each voltage-clamp protocol run. The density of inward I_{HERG} was calculated at [K⁺]o = 40 mM as the peak current elicited at a voltage of −120 mV after 20 s preconditioning at 0 mV, normalized by the cell capacitance. The values of the peak current refer to the current measured 10 ms after the heat-traces of the tracings obtained in the presence of 5 mM Cs⁺ or 1 μM WAY 123,398, according to Ref. 19. The I_{HERG} activation curves were measured according to Refs. 14 and 15. Resting potential (\(V_{\text{rest}}\)) was measured at 5 mM [K⁺]o in current-clamp mode (I = 0). The leakage conductance \(g_L\) was calculated at 5 mM [K⁺]o by a ramp protocol ranging from −100 to +60 mV and lasting 1280 ms, from a holding potential of 0 mV, as the slope of the current trace obtained in the presence of 5 mM Cs⁺ or 1 μM WAY 123,398, as previously described. The relative slow rate of voltage change produced a negligible capacitive current. The cell capacitance was obtained by directly reading the position of the amplifier knob of the cell capacitance compensation. Input resistance of the cells was in the range 2–6 GΩ. For data acquisition and analysis, pClamp software (Axon Instruments) and Origin (Microcal Software, Northampton, MA) were routinely used.

**RNA Extraction and Northern Blot—**Total RNA was extracted from FLG 29.1 cells by the guanidinium/soybean isoflavone method (28). Ten or 20 μg of total RNA were loaded on a formaldehyde-agarose gel and run at 80 mA for 3–4 h. Six micrograms of an RNA ladder (Life Technologies, Inc.) were also loaded. After staining with ethidium bromide, the gel was photographed and the position of the standards marked. RNA was then transferred by Northern blot onto a nylon membrane (Hybond N⁺; Amersham Pharmacia Biotech) and hybridized in Church Buffer (Na phosphate monobasic, 0.5 M, pH 7.2, 7% SDS, 1 mm EDTA) containing 10⁶ cpm/mg DNA of the appropriate probe (see below), at 65 °C overnight. Filter was then washed twice in Na phosphate 50 mM, SDS 1% for 5 min at room temperature, and once at 65 °C for 30 min, and exposed to x-ray film (Hyperfilm; Amersham Pharmacia Biotech) overnight at −70 °C for HERG probe, 5 min at −70 °C for 18S probe.

**Probes and Plasmids—**A 5′-Hi-HindIII full-length (3.5 kilobase) fragment of the HERG gene cloned in SP64 vector (29) kindly gifted by Dr. M. Keating (University of Utah, Salt Lake City, UT) was random priming labeled using [³²P]dCTP (Amersham Pharmacia Biotech), and the probe purified on Sephadex columns as described (28). An 18S probe...
Detection of Calcitonin Receptor by Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR)—Total RNA was extracted as reported above; cDNA was then synthesized from 1 µg of RNA using 200 units of reverse transcriptase SuperScript II (Life Technologies), plus 200 µM of each dNTP, 50 mM KCl, 0.5 µg of the specific primers (see below). Amplification was performed in a Robocycler (Stratagene), after an activation step at 94 °C for 2 min, for 30 cycles with 30 s at 94 °C (95 °C for the specific amplification of the CtR, using 2.5 units of Platinum Taq polymerase (Life Technologies), 200 µM of each dNTP, 1.5 mM MgCl₂, and 0.5 µM of the specific primers (see above). Amplification was performed in a Robocycler (Stratagene), after an activation step at 94 °C for 2 min, for 30 cycles with 30 s at 94 °C (95 °C for the specific amplification of the CtR, using 2.5 units of Platinum Taq polymerase (Life Technologies), 200 µM of each dNTP, 1.5 mM MgCl₂, and 0.5 µM of the specific primers (see above). The primers used were: 5’ sense, GCAGCTTCAGCTCATCGGCA; and 3’ antisense, CACCGTGCTGTTTAAACCCCT.

Immunoprecipitation of Integrins from Cell Surface Biotinylated Cells—Cells prepared as above were plated on ice and washed twice with Hank’s balanced salts (1.3 mM CaCl₂, 0.4 mM MgSO₄, 5 mM KCl, 138 mM NaCl, 5.6 mM d-glucose, 25 mM HEPES, pH 7.4) and biotinylated by adding 0.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce) in Hank’s buffer for 15 min. Cells were washed twice with Hank’s solution, labeling was repeated and the reaction was stopped by washing three times with serum-free Dulbecco’s modified Eagle’s medium + 0.6% BSA. Labeled cells were lysed in ice for 20 min with lysis buffer (Tris-HCl 20 mM, pH 7.4, NaCl 150 mM, glycerol 10%, Triton X-100 1%, phenylmethylsulfonyl fluoride 1 mM, aprotinin 0.15 units/ml, leupeptin 10 µg/ml, NaF 100 mM, Na vanadate 2 mM). The supernatant was then cleared by centrifugation at 16,000 g, removed by centrifugation at 3,600 g for 5 min, and then washed three times with ice-cold PBS and lysed on ice with 1% Triton X-100 containing the specific antibodies, suggesting the involvement of an integrin receptor containing the α₅ subunit in this adhesion. Since the polyclonal anti-FN-R antibodies contain a great amount of antibodies against epitopes belonging to the β₃ subunit, the most plausible explanation for this finding is that FLG 29.1 cells recognize VN

2 G. Hofmann, P. Delfilippi, and A. Arcangeli, unpublished results.

RESULTS

To study the relationships between ECM and integrin-mediated signals in FLG 29.1 leukemic cells, these cells were seeded on a FN-enriched substratum and the mechanisms sustaining cell adhesion were evaluated. In fact it had been previously shown that these cells could adhere to endothelial cells through the FN molecules covering endothelial cell surfaces (23) (see Introduction). Since FLG 29.1 cells express various classes of integrin receptors, mainly belonging to the β₃ and β₁ classes, and associated with different α subunits (α₅, α₅, α₅, α₅ and α₅) (23)² the role of these two different integrin classes on cell adhesion was evaluated. As shown in Fig. 1, about 50% of the FLG 29.1 cells adhere to FN, while this adhesion is significantly lower (about 35%) on VN, another integrin-recognized substrate. Cell adhesion to FN is impaired by blocking antibodies to FN receptor (α₅β₃), while unaffected by blocking antibodies to β₃ subunit (25), ruling out the involvement of β₃ subunit in leukemia cell adhesion to FN. As to cell adhesion to vitronectin, it is 40% inhibited by blocking antibodies to FN receptor, while only slightly affected by blocking antibodies to the β₃ subunit (see Fig. 1). However, FLG 29.1 adhesion to VN is impaired by RGD containing peptides, as well as by anti-α₅ antibodies, suggesting the involvement of an integrin receptor containing the α₅ subunit in this adhesion. Since the polyclonal anti-FN-R antibodies contain a great amount of antibodies against epitopes belonging to the β₃ subunit, the most plausible explanation for this finding is that FLG 29.1 cells recognize VN

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formed with antibodies against external epitopes of the three integrin subunits (not shown). Moreover, expression of $\alpha_v\beta_3$ integrin was 50% increased in FN-seeded cells (see inset to Fig. 3A and the legend to the figure), as compared with control BSA-seeded cells. This up-regulation is induced, although at a much higher extent, also by treatment with TPA (see lane c of Fig. 3A, and inset to the figure), indicating here again that the increase in $\alpha_v\beta_3$ integrin correlates with cell differentiation. As shown in panel B, another integrin expressed on FLG 29.1 plasma membrane, namely the $\beta_3$ subunit and its associated $\alpha(s)$, is not affected by adhesion of leukemic cells to FN. Moreover, a cytoplasmic protein, namely PKC$\alpha$, was not affected at all by cell adhesion to FN (panel C), ruling out any aspecific potentiating effect on protein synthesis operated by this adhesion.

On the whole, FLG 29.1 cells appear to be a suitable model for studying the electric signals induced by integrin activation, since these cells undergo a strong $\alpha_v\beta_3$-mediated adhesion to FN and this adhesion is capable of inducing the differentiation of these cells through the osteoclastic pathway, and, in this process, of sustaining an up-regulation of another integrin expressed in these cells, namely the $\alpha_v\beta_3$. The effect of integrin activation on various biophysical parameters was then studied in FLG 29.1, using the whole cell configuration of the patch clamp technique, after seeding the cells for different times on a FN- or a BSA-coated substratum, the latter taken as a control.

In Fig. 4 the time course of $V_{\text{REST}}$ is reported: this potential starts to be hyperpolarized in FN-seeded FLG 29.1 cells as soon as 15 min after seeding, reaching a value significantly different from that of the BSA-seeded control cells ($-28.6 \pm 2.6$ mV versus $-12.9 \pm 2.3$ mV; $p < 0.03$) after 1 h of incubation. Thereafter, $V_{\text{REST}}$ of FN-seeded FLG 29.1 cells returns to starting values, identical to that of control cells.

Since we previously demonstrated that only two currents contribute to regulate $V_{\text{REST}}$ in FLG 29.1 cells (12, 20), namely $I_{\text{HERG}}$ and the leakage, values relative to these parameters were evaluated and are reported in Table I: $I_{\text{HERG}}$ density is significantly increased after 1 h of cell adhesion to FN, as compared with the controls. This activating effect of FN on $I_{\text{HERG}}$ declines thereafter, so that the $I_{\text{HERG}}$ density in FN-seeded cells displays values even lower than those of control cells, after 2 h of incubation. This increase in $I_{\text{HERG}}$ density is not accompanied by variations in biophysical characteristics of the current, such as the activation or the deactivation properties (not shown). On the other hand, the leakage conductance is not significantly increased by cell adhesion to FN: in fact, this parameter is even higher in BSA-seeded cells after 1 h of incubation, while is not significantly different in the two experimental conditions after 2 h. This effect on the leakage conductance elicited by laminin in human neuroblastoma cells (13), where a concomitant activation of $I_{\text{HERG}}$ and leakage conductance is observed.

The integrin involvement in the mechanism underlying the FN-induced HERG channel activation was further evaluated at 1 h of incubation and results are reported in Table II: as shown in the table, the effect of FN is not unique, since also the adhesion to VN induces the activation of $I_{\text{HERG}}$ in FLG 29.1 cells, although at a slightly lower extent as compared with FN. This result suggests, even in this case as in neuroblastoma cells (13), the involvement of the $\beta_3$ subunit of integrin receptors in the increment of $I_{\text{HERG}}$: this is clearly proven by the fact that FLG 29.1 cell adhesion to dishes coated with mAb BV7 or TS2/16 to the $\beta_3$ subunit (24, 13) (bound anti-$\beta_3$-ab in Table II) induces a significant increase in $I_{\text{HERG}}$ density, whose intensity is even greater than that induced by adhesion on the whole FN...
molecule. Moreover, $I_{\text{HERG}}$ density was also increased at the same extent by antibody-induced clustering of $\beta_1$ integrin subunits on FLG 29.1 cells kept in suspension (soluble anti-$\beta_1$-ab in Table II). These data indicate that antibody-induced $\beta_1$ clustering is sufficient to trigger HERG channel activation. The specificity of the involvement of the $\beta_1$ subunit in HERG channel activation is confirmed by data, also reported in Table II, showing that dishes coated with antibodies directed against either an $\alpha$ subunit (anti-$\alpha_v$) or the $\beta_3$ subunit do not substantially increase $I_{\text{HERG}}$ density. On the whole, data reported in Fig. 4 and Tables I and II demonstrate that FLG 29.1 adhesion on FN induces a cycle of $V_{\text{REST}}$ hyperpolarization, which peaks after 1 h of incubation and is conceivably sustained by a concomitant increase in $I_{\text{HERG}}$, the latter being induced by the engagement of $\beta_1$ integrin subunit with its ligand.

We then analyzed whether the above reported increment in $I_{\text{HERG}}$ density could be reconstituted to variations in herg RNA level and/or modification of the HERG protein on the plasma membrane. In Fig. 5 the results of this analysis are reported. Fig. 5A shows a Northern blot performed with RNA extracted from cells seeded on BSA- or FN-coated dishes for 1 h, and probed with herg or a control gene (ribosomal 18S). It is evident that FLG 29.1 cell adhesion to FN is not accompanied by any variations in herg RNA level as compared with the controls. The effect of cell adhesion on HERG protein expression on the plasma membrane is shown in Fig. 5B, where an immunoblot performed on membrane proteins extracted from FLG 29.1 cells seeded on BSA or FN for 1 h, and revealed with an anti-HERG antibody is reported. Two protein bands are evident in FLG 29.1 membrane extracts, one upper band of 155 kDa, and a lower band of 135 kDa. According to data reported so far (32), the 155-kDa isoform represents the completely mature, fully glycosylated form of the protein, expressed on the plasma membrane, while the 135-kDa band is the immature, core-glycosylated protein. It is evident that FN adhesion does not alter this HERG protein profile, neither quantitatively, nor qualitatively.

Therefore, the FN-induced increment in $I_{\text{HERG}}$ could be realized by the action of a short range modulator of channel activity: a possible candidate could be envisaged in a pertussis toxin-sensitive $G_i$ protein interposed between $\alpha_v\beta_3$ integrin and HERG channel protein, as in neuroblastoma cells (11). And in fact, as shown in Fig. 5C, when FLG 29.1 cells are seeded on FN- (lane d) or activating anti-$\beta_1$ antibody-coated dishes (lane e), in the presence of PTX, no increase in $I_{\text{HERG}}$ density can be observed as compared with BSA-seeded control cells. It is worth noting that PTX-treated cells do adhere to FN, but do not spread on the substrate (panels c and c'), an effect already reported for PTX on melanoma cells (33). It is important to note that patch clamp experiments were performed either on adherent round or spread cells, and no difference in $I_{\text{HERG}}$ density as well as biophysical characteristics of the current were detected in the two conditions (not shown). This rules out that the lack of effect of FN on $I_{\text{HERG}}$ in PTX-treated cells can be due to the incapacity of the cells to undergo cell spreading.

The question now arises as to whether the FN-induced, $\beta_1$-mediated electric signal is a necessary step in the pathway leading to osteoclastic differentiation of leukemic cells. Since we showed that this pathway can be evidenced by at least three markers (CD 51/\(\alpha_\beta_3\) integrin, TRAP, and CtR receptor), ex-
of osteoclastic differentiation, cells were incubated in RPMI complete medium and incubated for different times. As a control FLG 29.1 cells.

Cells were seeded on BSA- or FN-coated dishes in integrin immunoprecipitated using a polyclonal antibody raised against the integrin α, Molecular weight markers (Bio-Rad) are reported on the right. In nonreducing conditions, the β1 integrin subunit migrates as an 80-kDa band (see arrow), while the associated α5 as a 150-kDa band. α5 also complexes with the β1 subunit, which migrates as a 110-kDa band. B, densitometric analysis of the amount of β1 band; the analysis gave the following results: control (BSA) = 1, FN = 1.5, TPA = 2.5 (indicated in arbitrary units). C, immunoprecipitation using a polyclonal antibody raised against the integrin α5. Molecular weight markers (Bio-Rad) are reported on the right. In nonreducing conditions, the β1 integrin subunit migrates as an 80-kDa band (see arrow), while the associated α5 as a 150-kDa band. α5 also complexes with the β1 subunit, which migrates as a 110-kDa band. B, densitometric analysis of the amount of β1 band; the analysis gave the following results: control (BSA) = 1, FN = 1.5, TPA = 2.5 (indicated in arbitrary units). C, immunoprecipitation using a polyclonal antibody raised against the integrin α5.

DISCUSSION

Integrin receptors are known to mediate either “inside-to-out” and “outside-to-in” signals (9), and, in this way, can regulate many cellular functions, such as cell growth and differentiation, cell migration and/or activation. We present evidence that β1 integrin receptors can elicit the activation of a hyperpolarizing, HERG-sustained, K+ current in leukemic hemopoietic precursors (FLG 29.1 cells), and that this signal apparently

FIG. 3. Effect of cell adhesion to FN on integrin expression on FLG 29.1 cells. Cells were seeded on BSA- or FN-coated dishes in RPMI complete medium and incubated for different times. As a control FLG 29.1 cells.

FIG. 4. Modulation of VREST of FLG 29.1 cells by cell adhesion. Cells from preparatory cultures were resuspended in RPMI + BSA and seeded on Petri dishes previously coated with the indicated substrates. At various times of incubation VREST was measured by the patch clamp technique (whole cell configuration; I = 0) (see “Experimental Procedures”). Values are mean ± S.E. of the number of cells reported in parentheses. Closed circles, BSA. Closed squares, FN.

TABLE I

Effects of cell adhesion on various biophysical parameters of FLG 29.1 cells at different times of incubation

Cells were seeded on BSA- or FN-coated dishes, and various biophysical parameters were studied at different times of incubation by means of the patch-clamp technique (see “Experimental Procedures”).

| Treatment | IHERGa density | t Test | gL | t Testa |
|-----------|----------------|--------|---|--------|
| BSA (1 h) | 28.9 ± 4.2 (16) | p = 0.0053 | 0.56 ± 0.09 (18) | NS |
| FN (1 h)  | 45.4 ± 3.5 (16) | 0.46 ± 0.19 (6) | 0.06 ± 0.13 (6) | NS |
| BSA (2 h) | 26.5 ± 5.2 (6)  | 0.46 ± 0.19 (6) | 0.06 ± 0.13 (6) | NS |
| FN (2 h)  | 17.4 ± 3.7 (6)  | 0.6 ± 0.13 (6) | 0.06 ± 0.13 (6) | NS |

a IHERG density was calculated by dividing the HERG peak current by the cell capacitance, gL, leakage conductance. Values are mean ± S.E. of recordings obtained from the number of cells reported in parentheses.
b p = Student’s t test (two populations).
c NS, not significant.

TABLE II

Effects of various substrates or anti-integrin antibodies on HERG current density

Cells were seeded on BSA-, VN-, or anti-integrin antibodies-coated dishes, or incubated with soluble anti-β1 antibodies, as explained under “Experimental Procedures”.

| Treatment | IHERGb density | t Testb |
|-----------|----------------|--------|
| BSA       | 28.8 ± 3.5 (22) | 0.034 |
| Bound anti-β1-ab | 53.1 ± 3.4 (14) | p = 0.005 |
| Soluble anti-β1-ab | 51.8 ± 8.9 (14) | 0.009 |
| Bound anti-β2-ab | 31.8 ± 4.1 (14) | NS |
| Bound anti-α5-ab | 25.0 ± 4.5 (5) | NS |

a IHERG density, calculated as reported in the legend to Table I, was measured after 1 h of incubation. Values are mean ± S.E. of recordings obtained from the number of cells reported in parentheses.
b p = Student’s t test (two populations).
c NS, not significant.
modulates the increased expression of $\alpha\beta_3$ integrins on the plasma membrane, a phenotypic marker of osteoclastic differentiation in these cells.

An integrin-mediated electric signal had been previously reported to occur in murine erythroleukemic (34), as well as murine and human neuroblastoma cells (11, 13). While in murine leukemic cells, adhesion to FN leads to the activation of $\mathrm{Ca}^{2+}$-dependent K$^+$ channels, the electric signal elicited by FN in FLG 29.1 leukemic cells is represented by a hyperpolarization of $V_{\mathrm{REST}}$, mediated by the activation of HERG K$^+$ channels, as occurs in neuroblastoma cells (11, 13). Therefore, HERG channels appear to be frequently involved in the signaling pathway induced by integrins in cells of different lineages.

An association between integrins and K$^+$ channels has been reported to occur between $\beta_1$ subunit and GIRK channels in transfected cells (35), although integrins do not appear to be essential for a functional GIRK expression (36), despite the physical association of the two proteins. A functional association between integrins and a K$^+$ channel different from HERG involves the Kv 1.3 channels in human lymphocytes: in these cells, however, the modulation of the K$^+$ current is the trigger for $\beta_3$ integrin activation, which leads to an enhanced functioning of the adhesive receptor (37).

In our case, the integrin-induced HERG activation does not imply any modulation in herg RNA or HERG protein level, but is mediated by a short-range mechanism of activation. In particular, in human leukemic, as in neuroblastoma cells (11), a $G_i$ protein is involved in the functional association between integrins and HERG channels. Integrins are known to modulate the activity of the Rho family of small GTPases (reviewed in Ref. 38), but also a physical and functional association between integrins, the integrin-associated protein and trimeric $G_i$ proteins has been reported (39, 40). These proteins constitute a signaling complex, involved in many cellular functions, such as chemotaxis (41) and platelet aggregation (39), possibly through the activation of $\beta_3$ integrins. In such models, the stimulation of $G_i$ induces an inhibition of adenyl cyclase, which ultimately leads to a decrease in intracellular cAMP. We have no evidence whether such a mechanism can also be invoked in our model, or whether a direct effect of $G_i$ occurs on HERG channels as reported for other types of K$^+$ channels (42).

Another point to be stressed here is that HERG channels, and their mediated electric variation of $V_{\mathrm{REST}}$, appear more frequently involved in processes of cell differentiation (11, 13, 43, 44). In fact, we show here that leukemic cells apparently start undergoing a process of osteoclastic differentiation, after adhesion to FN, as evidenced by the appearance of osteoclast markers, such as TRAP, CtR, as well as CD51/\(\alpha\beta_3\) integrin.

This is an important demonstration of a differentiating effect of the ECM on cells of the hemopoietic lineage, which couples with recent evidence showing a cooperative effect of FN with cytokines and growth factors on the maintenance and growth of CD34$^+$ hemopoietic stem cells (45).

Moreover, data reported in this paper demonstrate that this differentiation process includes the appearance of immunoreactivity to CD51, i.e. by the increased expression of $\alpha_\beta_3$ inte-
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increased function of β₂ integrins, possibly through a conformational change of the integrin molecule; however, an increased expression of this integrin subunit on the plasma membrane, as in our case, is not reported. While the effect of HERG channels on CtR appears to happen at the RNA level, the mechanism leading, in our model, to the up-regulation of β₂ integrins remains to be explored, and a transcriptional activation could be invoked. However, a direct electrostatic effect on the protein could also occur, thus candidating some classes of integrins as voltage-dependent proteins (46), capable of being influenced themselves by the biophysical modifications of the plasma membrane. On the whole, a mechanism of cross-talk between integrin subunits could be hypothesized, centered on the activity of K⁺ channels, which can be influenced by integrins (β₂ subunits, mainly), and can activate diverse proteins, either membrane-endowed, such as integrins themselves (β₂ subunits mainly), and hormone receptors, or cytoplasmic, such as pp125⁴⁷⁴ (47).

On the whole, these data support the hypothesis that HERG channels represent an important molecular device, involved both in the integrin-mediated outside-to-in and inside-to-out signaling and by this way in some signaling pathways controlling cell differentiation in the hemopoietic system.

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