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HERG potassium channels are constitutively expressed in primary human acute myeloid leukemias and regulate cell proliferation of normal and leukemic hemopoietic progenitors

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An important target in the understanding of the pathogenesis of acute myeloid leukemias (AML) relies on deciphering the molecular features of normal and leukemic hemopoietic progenitors. In particular, the analysis of the mechanisms involved in the regulation of cell proliferation is decisive for the establishment of new targeted therapies. To gain further insight into this topic we report herein a novel approach by analyzing the role of HERG K^+ channels in the regulation of hemopoietic cell proliferation. These channels, encoded by the human *ether-a-gò-gò-related* gene (*herg*), belong to a family of K^+ channels, whose role in oncogenesis has been recently demonstrated. We report here that *herg* is switched off in normal peripheral blood mononuclear cells (PBMNC) as well as in circulating $CD34^+$ cells, however, it is rapidly turned on in the latter upon induction of the mitotic cycle. Moreover, *herg* appears to be constitutively activated in leukemic cell lines as well as in the majority of circulating blasts from primary AML. Evidence is also provided that HERG channel activity regulates cell proliferation in stimulated $CD34^+$ as well as in blast cells from AML patients. These results open new perspectives on the pathogenetic role of HERG K^+ channels in leukemias.
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Keywords: AML; hemopoietic progenitors; $CD34^+$; HERG; *herg*; HERG inhibitors

Introduction

The demonstration that acute myeloid leukemia (AML) is a disease of hemopoietic stem cells has raised fundamental questions concerning the true target of leukemic transformation in this disease.[1] In this light, many studies have been focused on the identification and characterization of leukemic progenitor cell populations in patients with AML.[2–4] While the phenotype of leukemic stem cells has been defined,[2–7] it is emerging that primitive AML cells aberrantly express several set of genes, particularly those involved in the regulation of cell cycle progression and apoptosis.[8,9] Hence, the comparison between the pattern of genes expressed in normal *vs* leukemic progenitors is now regarded as a fundamental topic in the understanding of the biological features of malignant transformation leading to AML development.

A novel approach in this field is the study of the expression of ion channel encoding genes during cell cycle progression. In fact, recent studies suggested that ion channels, particularly K^+ channels, are important regulators of cell proliferation,[10] and it has been hypothesized that the activation of K^+ channels is required for the progression of cells through the G_1 phase of the cell cycle.[11–15] Moreover, the proliferation of normal and tumor cell lines[16–22] is often reduced and sometimes blocked by K^+ channel inhibitors. More recently, K^+ channel expression has been linked to the activity of well-known oncogenes[23] and a direct oncogenic potential has been attributed to K^+ channel encoding genes.[24] A possible link between K^+ channel activity and cell proliferation could be the clamping of the resting membrane potential (V_{REST}) value, usually afforded by these types of channels. Oscillations of V_{REST} in phase with the cell cycle transitions have been observed in various cell types, and particularly K^+-dependent hyperpolarizations at the G_1/S boundary.[10] On the other hand, while terminally differentiated, G_0 cells are usually hyperpolarized (with V_{REST} between −50 and −70 mV), cycling, and especially tumor cells tend to be depolarized.[27]

A contribution to explain this complex pattern came from our demonstration that, in a set of tumor cells, V_{REST} is often clamped to almost depolarized values by HERG K^+ currents (I_{HERG}). The *human eag-related* gene (*herg*) belongs to an evolutionary conserved multigenic family of voltage-activated K^+ channels, the *eag* (*ether a-gò-gò*) family,[26] and encodes for a channel contributing to the cardiac repolarizing current I_{Kr}.[27] I_{HERG} besides being a very tiny current, has profound physiological importance: in fact, in the cardiac model I_{Kr} regulates the repolarization phase of the action potential and its alterations lead to dramatic cardiac arrhythmias. Moreover, since the steady-state conductance of HERG channels is maximal around −30/−40 mV, I_{HERG} appears to be particularly suitable to the clamping of V_{REST} around these values in non-excitable cells, such as neoplastic or immature cells. Indeed, we showed that both *herg* and I_{HERG} are preferentially expressed in tumor cell lines of different histogenesis,[28,29] in primary human endometrial cancers,[30] as well as in neuroblasts and myoblasts only at very early stages of embryo development,[31,32] and determine the V_{REST} values in these cells.[28,29,33,34] Moreover, the human cell line FLG 29.1 derived from a patient with M5a type leukemia[35,36] expresses *herg* and a V_{REST} which is governed by a unique K^+ current, namely I_{HERG}, whose biophysical features keep this potential at strongly depolarized values.[37] In these cells I_{HERG} is modulated by integrin activation, and acquires the ability to regulate gene transcription leading to osteoclastic differentiation; in these conditions cells stop growing while undergoing a significant hyperpolarization of the V_{REST}.[38]

We present here a study on the expression and role of *herg* encoded channels in the hemopoietic system in both normal hemopoietic precursors and leukemic blasts present in AML. *herg* expression was first tested in resting peripheral mononuclear cells (PBMNC) obtained from normal donors as well as in $CD34^+$ collected from peripheral blood ($PBCD34^+$). The latter are more quiescent than $CD34^+$ cells of different origin (like cord blood $CD34^+$), but are able to respond to

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cytokine/growth factors stimulation and to enter into the mitotic cycle more rapidly than other CD34+ cells (eg bone marrow CD34+ cells).[39–41]

We report here that both PBMNC and PBCD34+ cells do not express *herg*, which is however rapidly up-regulated in the latter upon induction of proliferation by cytokines/growth factors. Moreover, this activation turned out to be necessary for CFU-GM proliferation *in vitro*. We also report that various myeloid leukemia cell lines as well as most human AML express *herg* and HERG channels in a definite subset of circulating blasts. Evidence is also reported demonstrating that HERG activity can control leukemia cell proliferation as well as the clonogenic potential of leukemic blasts present in the peripheral blood of AML.

On the whole, this study lends support to configuring *herg* as a proliferation-related gene, which is rapidly activated during cell cycle progression of human hemopoietic progenitors, while being constitutively expressed in immature leukemic cells.

Materials and methods

Isolation of peripheral blood mononuclear cells

Peripheral blood samples from 21 healthy donors were subjected to Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. Mononuclear cells were recovered at the interphase, washed and counted.

Preparation of CD34+ cells

Mononuclear cells obtained from pooled (4–8) peripheral blood buffy coats, obtained from blood donors were recovered from a Ficoll–Hypaque gradient, washed and counted. To select for CD34+ cells, about 10^8 mononuclear cells were applied to the Mini Macs column (Miltenyi Biotech, Gladbach, Germany), according to the manufacturer's instructions.

Isolation of leukemic blasts

Leukemic blasts were obtained from the peripheral blood of 46 patients (classified according to the French–American–British committee), treated either at the Hematology Unit of Firenze or at the Department of Internal Medicine of Torino, with newly diagnosed AML before chemotherapy. Enriched populations of blast cells (more than 95%) were obtained by Ficoll–Hypaque (Pharmacia) density gradient centrifugation. In some cases the cells were cryopreserved and stored in liquid nitrogen until use. The frozen AML cells were thawed, washed with RPMI 1640 medium (Hyclone, Logan, UT, USA), resuspended in IMDM (EuroClone Ltd, UK) medium containing 5% bovine calf serum (BCS) (Hyclone) at the concentration of 2×10^6/ml and incubated overnight at 37°C. After removal of clumped cells by sedimentation, the samples had a viability greater than 90%.

Cytofluorimetric analysis

Samples of 10^6 cells/ml were stained with propidium iodide (PI) as described by Vindelov and Christensen.[42] The samples were analyzed using a FACScan flow cytometer (Becton Dickinson, San José, CA, USA) equipped with a 5 W argon-ion laser. Histograms of cell number *vs* linear integrated red fluorescence were recorded for 50 000 nuclei/sample and were analyzed using the MultiCycle DNA content and cell cycle analysis software (Phoenix Flow Systems, San Diego, CA, USA) to evaluate the distribution in the phases of the cell cycle.

Cell culture and analysis of cell proliferation

The following leukemia cell lines were analyzed: FLG 29.1,[28] K562 (a cell line derived from the acute phase of a chronic myeloid leukemia[43]) and HL60 (a promyelocytic cell line[44]). Cells were cultured in RPMI with 5% heat-inactivated FCS (Hyclone Characterized, Logan, UT, USA) and maintained at 37°C in a humidified atmosphere in 5% CO_2 in air. For proliferation experiments cells were serum starved for 16 h in RPMI medium, then seeded in 24-well plates (Corning-Costar, Corning, NY, USA) at a cell density of 2×10^5 cells per well, in RPMI containing 1% FCS. When needed HERG channel blockers (CsCl 5 mM, E4031 40 μM and Way 123 398 40 μM, final concentrations) were added at time zero. After 24, 48 and 72 h, viable cells (determined by trypan blue exclusion) were counted in triplicate using a hemocytometer. Each experimental point represents the mean of four samples carried out in three separate experiments.

Clonogenic assay

AML blasts were seeded at a density of 1×10^6 in a 1 ml of mixture containing IMDM, 20% FCS, 10% agar and recombinant cytokines at the following concentrations: IL-3 10 ng/ml, GM-CSF 20 ng/ml, SCF 50 ng/ml, G-CSF 20 ng/ml. Each sample was then seeded in triplicate wells of a 24-well microplate in the absence or in the presence of the specific HERG current blocker Way 123,398 (200 μM, final concentration) and incubated at 37ºC in a humidified atmosphere in 5% CO_2 in air. After 10 days of incubation, all the aggregates containing more than eight cells were enumerated in each well and the results averaged.

CFU-GM assay

10^6 non-adherent mononuclear cells (MNAC) from normal peripheral blood were seeded as decribed above. Colonies and clusters were counted after 14 days of incubation.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Mononuclear cells were washed twice in ice-cold PBS and collected by centrifugation. Cells were homogenized in a guanidinium thiocyanate solution, and total RNA was extracted according to Cherubini *et al.*[30] RNA purity and integrity was always checked by running an aliquot on a denaturing 1% agarose gel.[45] cDNA was then synthesized from 1–2 μg of RNA using 200 U reverse transcriptase SuperScript II (Invitrogen, Groningen, The Netherlands), plus 200 μM each of dNTP and 2.5 μM random hexamers, in a 20 μl final reaction volume, for 50 min at 42ºC and 15 min at 70ºC. Two μl of cDNA were then amplified by polymerase chain reaction

in a 50 μl reaction containing Herculase enhanced DNA polymerase 2.5 U (Stratagene, La Jolla, CA, USA), 200 μM d(NTP)s, 1× Herculase polymerase reaction buffer. The oligonucleotide primers were as reported in Cherubini *et al*.[30] These primers encompass a nucleotide region from 2171 to 2746 of the human *herg* cDNA giving rise to a band 575 bp long. Thirty-five cycles of amplification were carried out after 2 min of enzyme activation at 94°C; denaturation was at 94°C for 30 s, annealing at 56°C for 1 min, and extention at 72°C for 1 min. Samples of PCR products were run on a 1.5% agarose gel using a molecular weight marker, the 100 bp DNA ladder (New England Biolabs, USA) and bands were visualized by ethidium bromide staining on a UV transilluminator. Control amplifications were performed adding either no DNA, no RNA and no retrotranscribed RNA in the PCR tube, but non-specific bands were never observed. cDNA samples were checked for integrity by PCR detection of human *gapdh* using the same conditions described above and using the same primers as reported in Cherubini *et al*.[30]

Immunocytochemistry

Leukemic cells were cytocentrifuged at 300 r.p.m. for 3 min and fixed in ethanol for 10 min at room temperature, and air dried before further analysis. Endogenous peroxidase activity was blocked by incubating the cells with 0.5% H_2O_2 in water for 10 min at room temperature. Before proceeding with the BioGenex Super Sensitive Detection System (BioGenex, San Ramon, CA, USA), cells were permeabilized with 0.2% Triton X-100 in Ultra V Block (Lab Vision, Fremont, CA, USA) for 20 min at room temperature. Slides were extensively rinsed with PBS and then incubated with the anti-HERG primary antibody (Alomone Labs, Israel), diluted 1:50 in a solution of 1:10 Ultra V Block/water. The anti-HERG antibody developed in our laboratory was diluted 1:50 as above. Further incubations were carried out for 20 min at room temperature: the first one with the prediluted biotinylated polyvalent anti-goat antibody, then with the horseradish peroxidase-conjugated streptavidin. After extensively washing with PBS, color was developed by incubating the slides with the DAB (3,3′-diamino-benzidine) chromogen solution for 5–40 min or until acceptable color intensity had been reached. Slides were then counterstained with Mayer's hematoxylin and mounted using Entellan mounting medium. Images were acquired through a Leica photocamera. Magnification was ×40.

Patch-clamp recordings

Cells plated on 35 mm Petri dishes were incubated at 37°C for appropriate times. Patch-clamp experiments were subsequently performed at room temperature with an amplifier Axopatch 2-A (Axon Instruments, Foster City, CA,USA). The whole-cell configuration of the patch-clamp technique[46] was employed using pipettes (borosilicate glass; Hilgenberg, Germany) whose resistance was in the range 2–3 MΩ. Gigaseal resistances were in the range 1–10 GΩ. Whole-cell currents were filtered at 2 KHz. For precise measurement of the gating parameters of the HERG channels, we carefully compensated pipette and cell capacitance and the series resistance before each voltage-clamp protocol run. Extracellular solutions were delivered through a nine-hole (0.6 mm), remote-controlled linear positioner placed near the cell under study. The standard extracellular solution contained (mM): NaCl 130, KCl 5, $CaCl_2$2, $MgCl_2$2, Hepes-NaOH 10, glucose 5, pH 7.4. The standard pipette solution at $[Ca^{2+}] = 10^{-7}$ M contained (mM): K^+ aspartate 130, NaCl 10, $MgCl_2$2, $CaCl_2$4, EGTA-KOH 10, Hepes KOH 10, pH 7.4. Cells were usually maintained in our standard solutions, until the whole-cell mode was obtained. Subsequently, the I_{HERG} was measured at $[K^+]_o = 40$ mM (substituted for NaCl), to magnify the current amplitude. The cell capacitance was obtained directly by reading the position of the amplifier knob of the cell capacitance compensation, and its mean value was 7.2 ± 1.2 pF ($n = 22$) for FLG cells. Resting potential (V_{REST}) was measured at 5 mM $[K^+]_o$ in open circuit conditions. The V_{rest} value of cells measured in conditions of active proliferation was -25 ± 3 mV ($n = 22$) for FLG 29.1 cells. For data acquisition and analysis, pClamp software (Axon Instruments) and Origin (Microcal Software, Northampton, MA, USA) were routinely used.

Results

Analysis of herg expression

herg expression was studied by RT-PCR in resting mononuclear cells collected from the peripheral blood (PBMNC) of different blood donors, as well as in the same samples pooled and enriched in CD34+ cells (PBCD34+). The latter was taken as representative of resting, G_0/G_1 hemopoietic progenitors.[39] As shown in Table 1 and Figure 1a (lanes 3 and 4) *herg* RNA was very rarely detected in PBMNC, while never detected in seven preparations of PBCD34+. Cytofluorimetric analysis performed on such PBCD34+ confirmed that the great majority (94.3%) of these cells was in the G_0/G_1 phase of the cell cycle (panel b in Figure 1), with only 1.5% in S and 4.1% in the G_2/M phase. However, when PBCD34+ were treated for 12 h *in vitro* with IL3, G-CSF and GM-CSF (see Materials and methods), cells entered the mitotic cycle (see panel c in Figure 1) with 25.3% of cells in S. In these cells *herg* mRNA expression was easily detectable (see panel a in Figure 1, lane 5). In contrast, no *herg* transcription was detected when cells were treated for shorter times (6 h) or on purified peripheral lymphocytes after phytohemoagglutinin stimulation (not shown). Thus, while *herg* is not transcribed in resting blood cells, nor in unstimulated CD34+ cells, it is up-regulated as soon as the latter are induced to enter the proliferative cycle by cytokine/growth factor stimulation.

Table 1 PCR analysis: number of normal controls and leukemia patients PCR positive for *herg* per number of studied subjects, and their relation to FAB type

PBMNC	2/21
PBCD34+	0/7
M0	1/2 (50)
M1	6/8 (75)
M2	9/12 (75)
M3	2/2 (100)
M4	12/14 (86)
M5	1/2 (50)
M6	3/3 (100)
M7	2/3 (67)
Total	36 /46 (78)

Values are the number positive/total number, with percentages in parentheses.

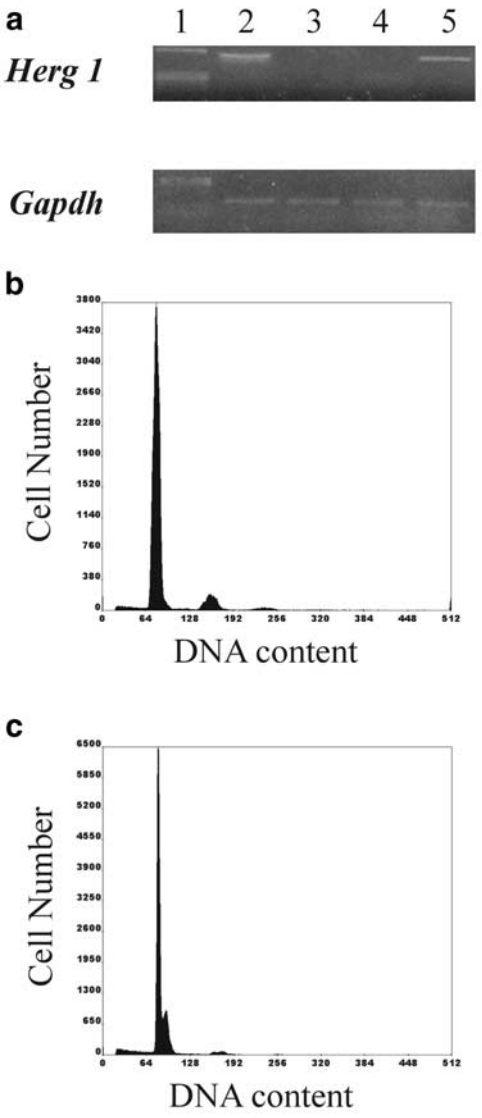

Figure 1 Expression of *herg* gene in PBMNC and PBCD34+ cells and its relation to the cell cycle. (a) RT-PCR relative to *herg* gene (upper panel, 575 bp band) and *gapdh* gene (lower panel, 138 bp band) in FLG 29.1 (lane 2), PBMNC (lane 3), PBCD34+ (lane 4) and PBCD34+ treated for 12 h *in vitro* with IL3, G-CSF and GM-CSF (lane 5). Lane 1: standard 100 bp (New England Biolabs). (b and c) Flow cytometry histograms of propidium iodide-labelled cells. (b) Resting PBCD34+ cells; (c) the same PBCD34+ cells after 12 h treatment with IL3, G-CSF and GM-CSF. Cell cycle analysis was performed as reported in Materials and methods. A representative experiment is reported.

Effects of HERG inhibitors on HSC and leukemic cell proliferation

The above results suggested the possible involvement of *herg*-encoded currents in regulating proliferation of hemopoietic progenitors. To address this question, the clonogenic activity of CFU-GM present in the PBCD34+ population was then evaluated in the absence or in the presence of the antiarrhythmic drug Way 123,398, a specific blocker of HERG K+ channels. As shown in Figure 2, CFU-GM expansion *in vitro* in semisolid medium was drastically blocked by Way 123,398.

These results prompted us to investigate whether HERG K+ channels were constitutively expressed in leukemic cells, in particular in AML, possibly regulating the uncontrolled neoplastic growth of these cells. Therefore, the expression of *herg* and HERG channels was first studied in myeloid leukemic cell lines obtained from human AML displaying different phenotypic markers. Although at different levels, *herg* mRNA was detected by RT-PCR not only in FLG 29.1 cells as previously reported[29] (Figure 3A, lane 2), but also in K562 and HL60 cell lines (Figure 3A, lanes 3 and 4, respectively). A typical I_{HERG} could also be detected in both K562 and HL60 cells: an example, relative to K562 cells, is reported in Figure 3B, where the biophysical and pharmacological features of I_{HERG} appear clearly similar to those reported in other tumor cell lines.[29]

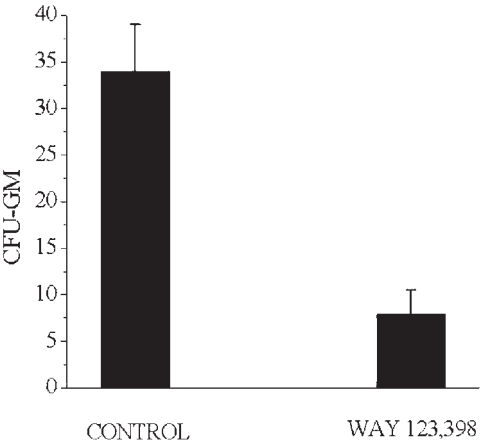

Figure 2 Effects of HERG inhibitors on colony growth from normal peripheral blood mononuclear non-adherent cells (MNAC). 10^6 non-adherent mononuclear cells (MNAC) from normal peripheral blood were seeded as described in Materials and methods in the absence (control) or in the presence of 200 μM Way 123,398. Colonies and clusters (representing CFU-GM) were counted after 14 days of incubation. Values are reported as the number of colonies and clusters (CFU-GM)/10^6cells/three wells, and are the average of two individual experiments.

To assess whether the activity of HERG channels affects the proliferation of leukemic cell population, as reported in different experimental models (see Introduction), the effect of HERG channels inhibitors (anthiarrhythmic drugs like E4031 and Way 123,398, or CsCl[44]) on this parameter was evaluated. As shown in Figure 4, when FLG 29.1 leukemia cells were starved by overnight serum deprivation, their subsequent proliferation after serum re-addition was inhibited by all the above mentioned HERG inhibitors, as well as by their combination. In the latter case, no strong additive effect was observed, indicating that all the inhibitors affected the same target, namely HERG currents. Moreover the same inhibitors did not affect the proliferation of cell lines not expressing I_{HERG} (Crociani *et al*, manuscript in preparation). The impairment of cell proliferation operated by HERG inhibitors is at least in part related to a retardation of cell cycle progression, as evidenced by the increase in the percentage of cells in the G_1 phase of the cell cycle observed in FLG 29.1 cells treated with the combination of HERG inhibitors (inset to Figure 4) after 24 h of incubation in 1% FCS. Substantially similar results were obtained in K562 (not shown). It is worth noting that in these experiments no increase in cell death via the apoptotic pathway was observed after treatment of cells with HERG inhibitors, either in the cytofluorimetric analysis or performing the Hoechst test (not shown).

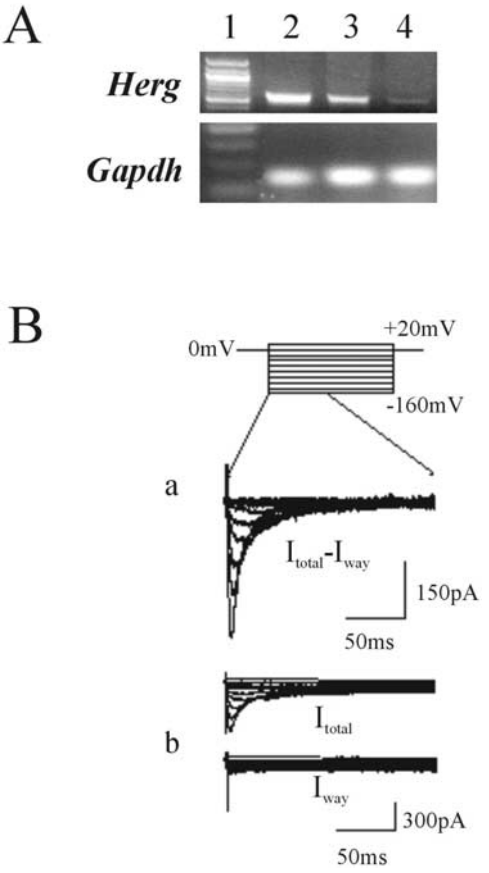

Figure 3 Expression of *herg* gene and HERG current in leukemia cell lines. (A) RT-PCR relative to *herg* gene (upper panel, 575 bp band) and *gapdh* gene (lower panel, 138 bp band) in FLG 29.1 (lane 2), K562 (lane 3) and HL60 (lane 4) cell lines. Lane 1: standard 100 bp (New England Biolabs). (B) HERG current traces obtained in K562 cells. I_{HERG} was measured at $[K^+]_o = 40$ mM, as the peak current elicited at voltages ranging from +20 to −140 mV, after holding the cell for 10 s at 0 mV. Upper panel: protocol used in the presented experiment: (a) traces obtained after subtracting from traces in control conditions, traces obtained after perfusion of the cell examined in the presence of 1 μM WAY 123,398 (both traces are reported in (b)).

On the whole, data presented so far suggested that HERG channels are involved in the regulation of cell cycle progression of various types of leukemia cell lines.

Expression of herg transcript and protein in primary AML cells

Experiments were then performed to study *herg* and HERG protein expression in primary human AML cells, as well as their role in leukemia cell proliferation. *herg* expression was evaluated by RT-PCR in a set of peripheral blood leukemic blasts from 46 patients with *de novo* AML at diagnosis. A representative example of a RT-PCR analysis is shown in Figure 5. Table 1 shows the complete results of our analysis and gives the rates of *herg* expression according to the FAB type.[35] Collectively, *herg* appears to be expressed in 36/46 (78%) of the AML examined, with the highest incidence in the M1, M2, M3 and M4 group; FAB type M2 and M4 accounted for the majority of the patients examined.

HERG protein expression was also investigated in leukemia cell lines and some samples of AML with immunocytochemistry, using the peroxidase method. In Figure 6, photomicrographs of immunocytochemistry of FLG 29.1 cells (panel A) and of a representative AML patient (panel B) are shown. In this latter case similar results were obtained both with a commercial anti-HERG antibody and the anti-HERG antibody developed in our laboratory (inset b), giving the same results. As evident in Figure 6, while all FLG cells resulted in positive staining, only some individual cells were positive for HERG immunostaining in the AML patient. Identical results were obtained in the other AML samples processed for immunocytochemistry (not shown). The immunocytochemical results

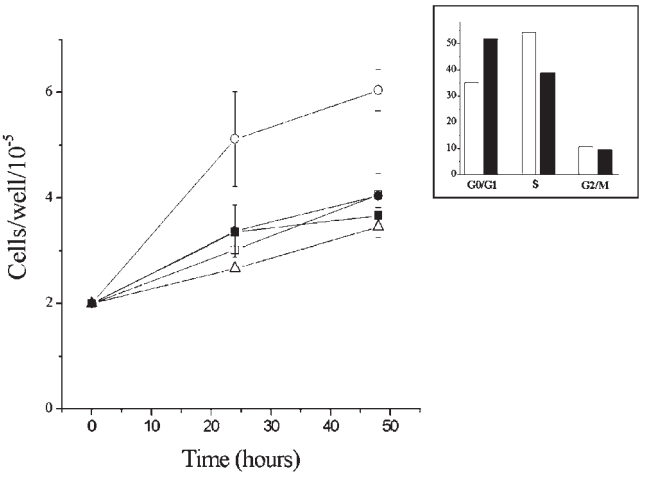

Figure 4 Effect of current inhibitors on proliferation and cell cycle progression of FLG 29.1 cells. Cell growth of FLG 29.1 in the absence and in the presence of HERG inhibitors. FLG cells were serum starved overnight, and then cultured in 1% FCS in the absence (open circles) or in the presence of 40 μM WAY 123,398 (closed circles), 40 μM E4031 (open squares), 5 mM CsCl (closed squares), or a mix of the three inhibitors (triangles). The high concentrations of HERG inhibitors were necessary due to the serum binding capacity for various types of drugs.[10] Inset: cell cycle analysis of FLG cells cultured for 24 h in the absence (white bars) or in the presence of HERG inhibitors (black bars). Values reported are the percentage of cells in the various phases of the cell cycle obtained in a representative experiment.

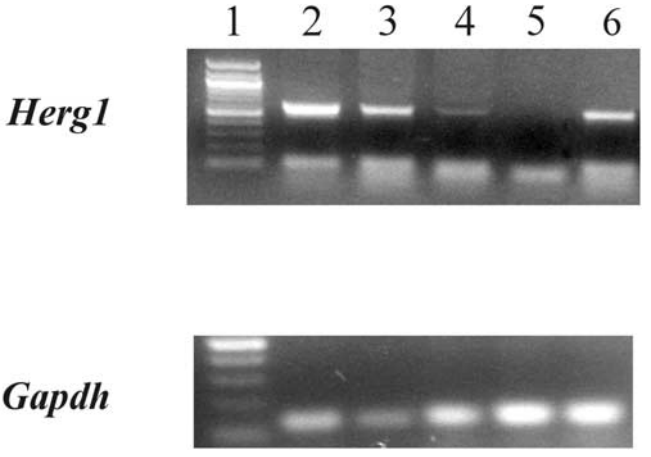

Figure 5 Representative RT-PCR of *herg* gene in primary AML and PBMNC from normal blood. RT-PCR relative to *herg1* gene (upper panel, 575 bp band) and *gapdh* gene (lower panel, 138 bp band) in FLG29.1(lane 2), patient with AML M2 (lane 3), patient with AML M1 (lane 4), PBMNC from normal donors (lane 5), patient with AML M4 (lane 6). Lane 1: standard 100 bp (New England Biolabs).

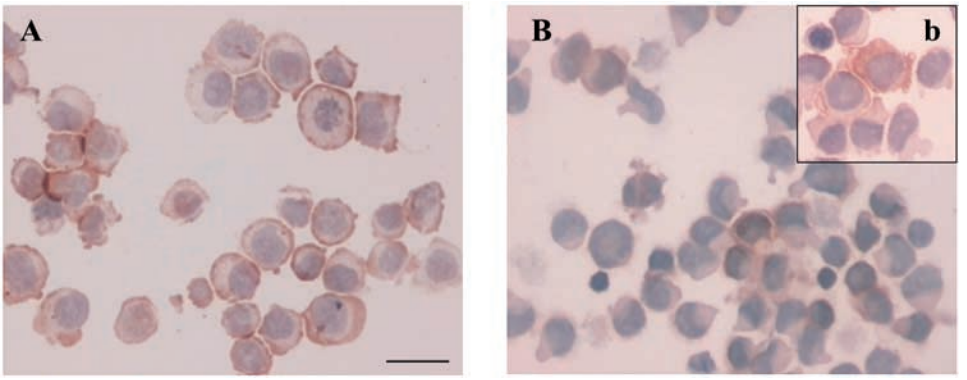

Figure 6 HERG protein detection in leukemic cell line FLG 29.1 (A) and in primary leukemic cells (B). Cells were immunostained with an anti-HERG primary antibody (Alomone Labs) directed against the C-terminus of HERG following the protocol reported in Materials and methods; (inset b) the same primary leukemic cells reported in B were immunostained with an anti-HERG antibody developed in our laboratory[20] and directed against the N-terminus of the same protein; bar 30 μm.

suggested that the expression of HERG protein can be easily detected by this method and that such expression is limited to definite cell types among the blast cell population.

To test whether HERG channel expression in primary AML could be involved in the regulation of cell proliferation, as in the above reported leukemia cell lines, a clonogenic assay was performed in some of the AML blasts examined in Table 1, in the absence or in the presence of the specific HERG channel inhibitor Way 123,398. A strong inhibition of colony formation was observed in almost all cases examined as compared to control, when cells were seeded in semisolid medium in the presence of Way 123,398 (Figure 7).

Discussion

In this paper we provide evidence that, at variance with mature PBMNC or resting PBCD34+ cells, growth factor-stimulated CD34+ cells and AML blasts express the K+ channel encoding gene *herg*, and that the progression along the mitotic cycle of hemopoietic progenitors cells, either normal or leukemic, is strongly affected by the activity of HERG K+ channels.

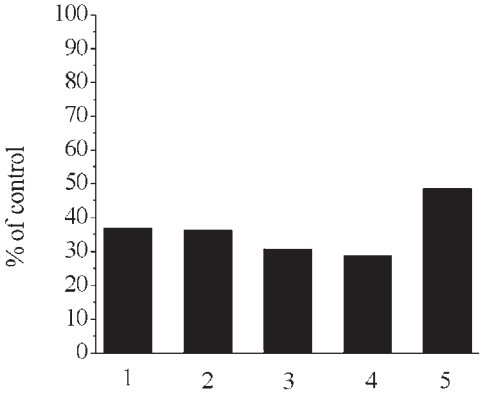

Figure 7 Effect of specific HERG channel inhibitors on colony formation from AML peripheral blood. The clonogenic assay was performed as reported in Materials and methods on AML blasts in the presence or absence of the HERG current inhibitor Way 123,398 (200 μM final concentation). The use of concentrations of Way 123,398 much higher than those used in experiments reported in Figure 4 was necessary due to the possible depletion/degradation of the drug over the large course of experiment. Values are reported as percentage of the number of colonies detected in treated cultures as compared to control, untreated cultures. Five different AML cases are reported.

The expression of *herg* was almost undetectable in PBMNC, and even in PBCD34+, that are in resting hemopoietic progenitors cells circulating in the bloodstream, but was capable of undergoing rapid activation of the mitotic cycle upon treatment with a cytokine/growth factor mixture. Indeed, PBCD34+ cells enter into the S phase as soon as 12 h after this treatment, and rapidly express the *herg* gene at that time. Many recent reports demonstrated that definite sets of genes, including those encoding growth factors and their receptors, cytokines and relative receptors, as well as transcription factors like c-*myc*, c-*myb*, c-*fos* and *c-jun*, are modulated in their transcription during response to cytokines/growth factors.[48] The results reported here demonstrate that the *herg* gene can be included in the set of genes activated early on during the G_0/G_1 to S transition of hemopoietic progenitor cells. It is worth noting that recent reports demonstrated that inward rectifier K+ (IRK) channels are up-regulated in cord blood CD34+ cells stimulated to enter cell cycle by a cytokine/growth factors mix.[49,50] In particular, the IRK current was detected in resting cord blood CD34+, and was expressed by a greater percentage of cells after 16 h incubation with IL3 and stem cell factor (SCF). We also detected the IRK current encoding gene, *Kir 2.1* in PBCD34+ before cytokine stimulation (data not shown). these data underline a profound difference between *herg* and *Kir 2.1* genes in that only the former is turned on upon cytokine stimulation at the onset of cell cycle progression.

Results reported in this study also demonstrate that *herg* is expressed in myeloid leukemia cell lines and in the majority of AML cases studied. Since cells in leukemic lines are randomly distributed throughout the mitotic cycle, it is conceivable that the *herg* gene is constitutively expressed in these cells independently of their cell cycle status or any stimulation. By contrast, in AML cases *herg* is apparently expressed in a definite set of cells (see immunocytochemical experiments), consistently with the finding that only a small proportion of blasts are able to divide, most of them being terminal cells.[51] Many recent reports aimed to define the characteristics of leukemic progenitor cells present in AML have shown the constitutive activation of transcription factors like STATs[52] or NF-kB,[9] as well as aberrant expression of tumor suppressor genes IRF1 and DAPK.[8,53] Data reported in this paper indicate the *herg* gene as the first K+ channel enco-

ding gene which is constitutively up-regulated in AML blasts, being completely turned off either in mature myeloid cells and in resting CD34+ cells.

We also show that HERG channels could be proposed as regulators of immature hemopoietic cells proliferation. In fact, the specific block of I_{HERG} in leukemia cell lines always impaired cell proliferation, irrespective of the drug's mechanism of action,[54] while not affecting growth in cell lines not expressing I_{HERG}. The antiproliferative effect can be accounted for by an increase in the number of cells present in the G_1 phase of the cell cycle. Moreover, we show here that the clonogenic potential of either stimulated CD34+ cells or circulating blasts of AML can be drastically reduced through specific HERG channel inhibition.

We have previously shown that HERG channels are linked to the cell cycle phases in NB cells,[28] as well as that a particular HERG protein isoform is up-regulated in the S phase of these cells (Crociani *et al*, manuscript in preparation). In the present paper we provide strong evidence that HERG channel activity is necessary for the progression of leukemic cells beyond the G_1/S boundary.

Previous reports indicated K+ channels as important regulators of leukemia cell proliferation, suggesting that the activation of such channels is a prerequisite for the G_1/S progression of cells.[10] The present paper offers a molecular dimension to this aspect, demonstrating that in activated PBCD34+ cells, leukemia cell lines and primary AML, cell proliferation is regulated by HERG channels.

Data here reported can also explain the preferential and constitutive *herg* expression in tumor cells. In fact, in these cells the main role of HERG currents could be that of regulating cell cycle progression, probably by clamping the V_{REST} values around –30/–40 mV. These are the values often directly measured in tumor cells,[25,28,29,33,34,38] as well as in leukemic cell lines studied in this paper (see Materials and methods). The latter could be a threshold value, required for cell survival, by preventing cells from losing K+ ions, a prerequisite to enter the apoptotic pathway.[55,56] Alternatively, such values could be apt to maintain, in a properly activated state,[57–59] those voltage-dependent proteins involved in cell signalling, as well as to control the appropriate Ca^{2+} entry into the cells through voltage-dependent channels.[60]

On the whole, based on data reported in this study, we propose to include K+ channel encoding genes, in particular the *herg* gene, among novel molecular markers of hematological disorders that are involved in the regulation of hemopoietic cell proliferation.

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