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K^+ channel antisense oligodeoxynucleotides inhibit cytokine-induced expansion of human hemopoietic progenitors

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Abstract Primitive human hemopoietic progenitor cells identified by surface membrane markers $CD33^-CD34^+$ are capable of expansion into lineage-restricted precursors following in vitro stimulation by hemopoietic regulators such as stem cell factor (SCF) and interleukin-3 (IL-3). In search of ionic currents involved in cytokine-induced progenitor cell growth and differentiation, human umbilical cord blood $CD33^-CD34^+$ cells were subjected to perforated patch-clamp recordings following overnight incubation with SCF and/or IL-3. An inward rectifying potassium channel (K_{ir}) was found in 33% of control unstimulated cells, in 34% of cells incubated with IL-3, in 31% of cells incubated with SCF and in 75% of cells incubated with IL-3 plus SCF. K_{ir} activity increased with elevation of extracellular potassium and was blocked by extracellular Cs^+ or Ba^{2+}. Antisense oligodeoxynucleotides directed against K_{ir} blocked both mRNA and functional expression of K_{ir} channels. K_{ir} antisense also inhibited the in vitro expansion of cytokine-stimulated $CD33^-CD34^+$ cells into erythroid (BFU-E) and myeloid (GM-CFU) progenitors in 7-day suspension cultures. Extracellular Cs^+ or Ba^{2+} induced a similar degree of inhibition (40–60%) of progenitor cell generation. These findings strongly suggest an essential role for K_{ir} in the process of cytokine-induced primitive progenitor cell growth and differentiation.

Key words Potassium channel physiology · Cell differentiation · Cytokine physiology · Hemopoietic progenitor physiology · Oligodeoxynucleotides antisense

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Introduction

Potassium (K) channel activity has been linked to cellular proliferation and differentiation in various systems, such as neurons [34], myocytes [32], epidermal cells [25] and fertilized oocytes [11]. Differentiation of hemopoietic cells is also associated with changes in K channels [9, 10, 24, 35]. For example, immature promyelocytic HL60 cells contain a slow-inactivating K channel, whereas HL60 cells induced into monocytic differentiation express an inwardly rectifying K channel, modulated by macrophage colony stimulating factor [35]. The link of K channels to cellular proliferation has also been described in human myeloblastic leukemic cells (ML-1). In these cells, decreased proliferation following induction of differentiation results in a reduction of K channel activity [24]. Lymphocytes have been found to contain 4-aminopyridine-sensitive voltage-gated K channels, whose activity has been linked to mitogen-induced stimulation of proliferation [10]. Beyond these acute changes in single-channel properties, a slow increase in the total K^+ current due to an increase in channel number occurs long after mitogenic or interleukin-2 (IL-2) stimulation of T-cells [6]. A variety of agents that block K channels inhibit DNA synthesis, as demonstrated by their inhibitory effect on human T-cell proliferation in response to lectins, IL-2 or alloantigens [4]. While the above-mentioned studies demonstrate the expression of K

channels including inward rectifiers in hemopoietic cells, it is not known whether these channels are *essential* elements in the process of hemopoietic cell growth and differentiation.

We have chosen to investigate this issue in the process of cytokine-mediated in vitro expansion of human hemopoietic progenitors which express the membrane antigens $CD33^-CD34^+$. Constituting 0.2–0.4% of bone marrow or cord blood mononuclear cells [1, 23], this primitive progenitor cell population gives rise to early erythroid (BFU-E) and granulocyte-macrophage (GM-CFU) progenitors, when stimulated by the cytokines interleukin-3 (IL-3) and stem cell factor (SCF) [8]. Although SCF alone does not induce hemopoietic progenitor cell growth in vitro, its synergistic effects with IL-3 have been well documented [2, 3].

In order to evaluate the involvement of K channels in the process of cytokine-induced progenitor cell expansion, we utilized perforated patch-clamping methodology [17] to record K^+ currents in isolated cord blood $CD33^-CD34^+$ cells stimulated with IL-3 and/or SCF. Here we report on a cytokine-dependent increase of an inward rectifier (K_{ir}) in these cells. To assess whether K_{ir} is essential for progenitor cell generation we employed an antisense oligodeoxynucleotide to inhibit channel expression and ion channel blockers to block K^+ current.

Materials and methods

Isolation of cells

Umbilical cord blood samples taken during delivery were fractionated on Ficoll-Hypaque and buoyant (< 1.077 g/cm^3) mononuclear cells were collected. The cells were subjected to indirect panning following incubation with anti-CD33 monoclonal antibodies (Coulter, Fla., USA). $CD33^-$ cells were collected, incubated with anti-CD34 antibodies (Caltag Lab, Calif., USA) and isolated by positive immunomagnetic bead (Dynal) selection, using a ratio of 0.025 beads/cell, resulting in $CD33^-CD34^+$ cells coated with one to three beads per cell [27]. In order to test the panning purification efficacy, $CD33^-$ cells were reanalyzed by FACS using CD33 as the first antibody. The results showed that the fraction of $CD33^+$ cells is below the detectable range. Immunomagnetic beads did not bind to control $CD33^-$ cells incubated in the absence of mouse anti-human CD34 antibodies, thereby verifying that no "false-positive" $CD34^+$ cells were isolated.

Patch-clamping

Following 1–24 h of incubation in Iscove's Modified Dulbecco's Medium (IMDM), in the presence or absence of cytokines, cells were washed and resuspended in a recording solution containing (in mM): NaCl 140, KCl 7, $MgCl_2$ 2, $CaCl_2$ 2, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, glucose 5. Ion currents were recorded in perforated, whole-cell and cell-attached patch-clamp configurations [15, 17]. An Axopatch 200 amplifier (Axon Instruments, Calif., USA) and Jencons H15 glass fabricated patch electrodes were used for all recordings. Data were acquired with a Digidata A/D board running under Axotape and pClamp software (Axon Instruments). Amphotericin B was used to obtain the perforated-patch configuration which was employed to prevent rundown of any current under cellular modulation [17, 31]. Isolation and identification of different currents was achieved with the blockers Cs^+, Ba^{+2}, tetraethylammonium (TEA), charybdotoxin (generous gift of C. Miller), and 4-aminopyridine (4-AP). Cell surface area was calculated from whole-cell capacitance measurements utilizing a triangle wave form under voltage-clamp and based on a 1 μF/cm^2 membrane capacitance. Recordings were carried out at room temperature (21° C). The criterion for defining K_{ir}-positive vs K_{ir}-negative cells is based on a conductance ratio measurement in the −50 to −30 mV and −110 to −90 mV ranges. K_{ir} is activated only in the second voltage range (see Fig. 1A). The ratio values show a distinct bimodal distribution constituting two populations (F test, $P < 10^{-37}$) separated at a ratio value of 0.4. Cells with a ratio below 0.4 were defined as K_{ir}-negative and above 0.4 as K_{ir}-positive. The latter had a mean ratio of 1.91 ± 0.22 and the former 0.10 ± 0.01. Detection resolution is about five channels based on a measured current peak to peak noise level of 10 pA at −110 mV and a single-channel conductance of 60 pS (result not shown).

In vitro expansion of $CD33^-CD34^+$ cells into committed myeloid and erythroid progenitors

Following isolation, $CD33^-CD34^+$ cells were incubated at 2×10^3/0.1 ml culture consisting of Modified Dulbecco's Medium (MDM) supplemented with 10% heat inactivated fetal calf serum (FCS) (Beit Haemek Biological Industries, Israel), 10 mg/ml bovine serum albumin (BSA, Sigma), 4×10^{-6} M iron-saturated transferrin (Sigma), 1×10^{-8} M sodium selenite (Sigma), 10^{-4} M 2-mercaptoethanol (Sigma), 1 g/l nucleosides (Sigma) and 1.5×10^{-5} M each of linoleic acid and cholesterol (Sigma) in the presence or absence of 5 ng/ml recombinant human (rh) IL-3 (Genetics Inst., Cambridge, Mass., USA) and 40 ng/ml rhSCF (AmGEN, Thousand Oaks, Calif., USA). Following 7 days of incubation at 37° C in a fully humidified incubator containing 7.5% CO_2 in air, cells were collected, washed and resuspended for colony growth. For these assays cells were cultured in the above-described medium, using 0.9% methylcellulose as viscous support. The growth of erythroid progenitors (BFU-E) was stimulated by 5 ng/ml IL-3 and 2U/ml recombinant human erythropoietin (AmGEN). Myeloid (GM-CFU) progenitors were grown in the presence of IL-3 alone. Colonies were enumerated following 14 days of methylcellulose culture.

The degree of progenitor enrichment was based on the number of colonies formed in methylcellulose cultures by $CD33^-CD34^+$ cells plated immediately after isolation (day 0) compared to number of colonies generated by cells plated after a 7-day incubation treatment (day 7).

Antisense oligodeoxynucleotide design

An 18-bp-long oligodeoxynucleotide was synthesized (Bio-Technology General, Rehovot, Israel) based on the published sequence of the cloned K_{ir} (IRK1) from mouse macrophages [22] and corresponding to the aminoterminal domain (aa 4–9). The antisense sequence is 5′GTAGCGGTTGGTTCTCACGCGAA-AGC3′ and the scrambled sequence used is 5′GATCGACTCTG-GTGCTGTGCGAAAGC3′. Both include an 8-bp sequence added to the 3′ end of the oligodeoxynucleotides in order to fold into a hairpin structure which stabilizes oligodeoxynucleotides in serum-containing media [21]. The oligodeoxynucleotides were checked for the absence of internal hairpin regions and complementary regions to other known relevant cloned human genes (the GenEMBL database was used utilizing DNasis software). Prior to addition to the cells, the oligodeoxynucleotides were boiled for 10 min and cooled slowly to room temperature allowing the hairpin to fold. The antisense or scrambled oligodeoxynucleotides was added (100 μg/ml) to $CD33^-CD34^+$ cells at the initiation of 7-day

suspension cultures. No specific effect was observed in single dose experiments employing antisense and scrambled oligodeoxynucleotide concentrations of 20 and 50 μg/ml.

Reverse transcriptase-polymerase chain reaction

RT-PCR was used for detection of the K_{ir} gene transcript [12]. Briefly, total RNA was extracted from pooled samples constituting 0.5×10^6 $CD33^-CD34^+$ cells by the phenol acid technique [5] and 1 μg was subjected to reverse transcription using random hexamere primers. The cDNA was then subjected to PCR analysis. Cycling parameters: denaturation at 94° C, 1 min, annealing at 50° C, 1 min, and extension at 72° C, 1 min for 32 cycles. A degenerated upstream oligonucleotide [5′CCATT/AGGT/CTAC/TGGA/-TTTCAGG3′] corresponding to the conserved sequences of the H5 pore domain of the recently cloned IRK1 and ROMK1 [15, 22] and a downstream primer [5′GGAGATATGACTGGCTGA3′] spanning the stop codon of the IRK1 channel cDNA were used to amplify an approximately 865-bp cDNA fragment from human blood $CD33^-CD34^+$ cells treated with or without either a control scrambled or antisense oligodeoxynucleotide. Semi-quantitative PCR was carried out to quantify the input mRNA and related cDNA of the control and treated human stem cells. The coamplification of an internal control housekeeping human S14 ribosomal protein mRNA was performed using upstream [5′GGCAGACCGAGATGAATC3′] and a downstream primer [5′CAGGTCCAGGGGTCTTGG3′] which amplify a 143-bp cDNA fragment. An equal aliquot of each PCR product was removed at various cycle numbers (cycle no: 16, 20, 24, 26, 28, 30, 32] and analyzed by 1.2% agarose gel electrophoresis after staining with ethidium bromide.

HL60 cell proliferation

Human promyelocytic leukemic HL60 cells were cultured in triplicates in 96-well dishes at 10^5 cells/ml. Cells were grown in MDM as described above for colony assays with omission of FCS, in the absence or presence of an antisense oligodeoxynucleotide. Following 48–72 h (approximately two cell division cycles), cell numbers were determined by MTT {3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue} reduction [13].

Immunofluorescence

Cells were incubated at 1×10^6/0.1 ml at 4° C for 30 min, with a saturating concentration of a monoclonal antibody that recognizes the CD33 cell determinant (Coulter Corp, Fla., USA). The cells were washed twice in ice-cold phosphate-buffered saline containing 5% heat-inactivated FCS (Gibco Laboratories, Grand Island, N. Y., or Beit Haemek Biological Industries, Israel), followed by an additional 30-min incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) (Sigma, St. Louis, Mo., USA). Controls were incubated with the second antibody alone. Immunofluorescence was determined using a Beckton-Dickinson Flow Cytometer, following an initial gating with forward and side scatter to eliminate debris and to identify a uniform population of cells. Results of CD33 labeled cells are expressed as a percentage of cells with a fluorescence intensity above the maximal fluorescence intensity of cells labeled with the second antibody alone.

Statistics

Results are given as $N \pm$ standard error of the mean. Unless indicated otherwise Student's t-test was used to test for statistical significance.

Results

Expression of K_{ir} in primitive hemopoietic progenitors

Perforated patch-clamp recordings of isolated cord blood $CD33^-CD34^+$ cells cultured for 16 h revealed an inward rectifying K^+ current (K_{ir}) in 33% of unstimulated cells tested ($n = 29$) (Fig. 1A). The current exhibited characteristic features of K_{ir} channel current as seen in the current/voltage curve (Fig. 1B). The inflection point was around -70 mV close to the calculated K reversal potential (-75 mV). Depolarizing the membrane by approximately +20 mV induces a small outward current. In the hyperpolarizing region, the current conductance is markedly dependent on external K concentration ([K].) (Fig. 1B). The mean K_{ir} conductance in the -120 to -80 mV range, at $[K]_o = 7$ mM, was 0.45 ± 0.14 nS/pF ($n = 10$). K_{ir} was further characterized by sensitivity to K channel blockers. K_{ir} was blocked by external application of 1 mM Cs^+ ($n = 36$) or 2 mM Ba^{2+} ($n = 9$) (Fig. 1C). The current was insensitive to 30 mM TEA ($n = 5$), 10 mM 4-AP ($n = 5$) and 100 nM charybdotoxin ($n = 5$).

Effect of cytokines on K_{ir} expression

To examine the involvement of K_{ir} channels in cytokine-induced progenitor cell expansion, we first analyzed and compared the effect of two early-acting hemopoietic regulators on K_{ir} expression in primitive hemopoietic progenitors. In preliminary experiments, acute application of 5 ng/ml IL-3 ($n = 4$), SCF 40 ng/ml ($n = 11$) or both ($n = 9$) during whole-cell recordings did not induce any detectable changes in current levels for periods of up to 30 min. Since these cytokines are mutually enhancing, the application of IL-3 or SCF for 30 min was preceded by an overnight incubation with the complementary (SCF or IL-3) cytokine. This was repeated under perforated patch-clamp recording conditions to avoid possible dilution of cytoplasmic messengers involved in signal transduction processing triggered by the cytokines. Since no change in current was observed under these conditions as well, we resorted to a prolonged incubation period prior to recording. Isolated $CD33^-CD34^+$ cells were incubated for 16 h with IL-3 and SCF, alone or in combination. In unstimulated cells K_{ir} was expressed in 10/29 (33%) cells (Fig. 2), the percentage of cells expressing K_{ir} following incubation with IL-3 (9/27; 34%) or with SCF (6/19; 31%) remained unaltered. However, a significantly higher percentage of cells was found to express K_{ir} following incubation with both IL-3 and SCF (15/20; 75%). The K_{ir} conductances were variable but no significant differences ($P > 0.3$) were detected between K_{ir}-positive cells under the different treatments (IL-3 0.63 ± 0.12 nS/pF $n = 9$, SCF 0.47 ± 0.05 nS/pF

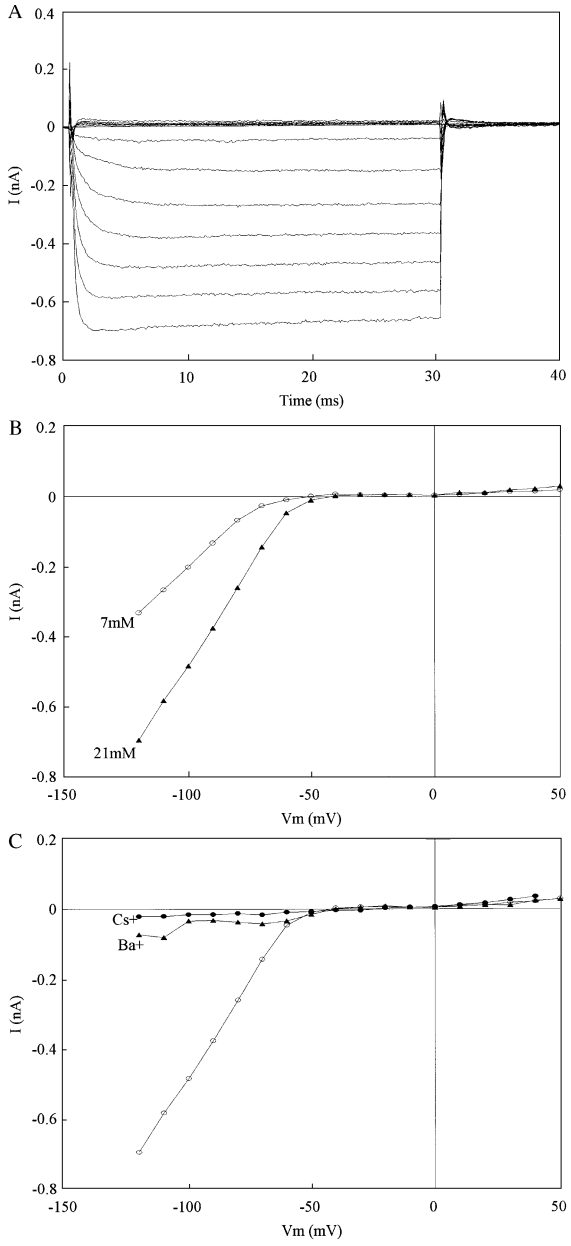

Fig. 1A–C Inwardly rectifying $K(K_{ir})$ channels from $CD33^-CD34^+$ cells. **A** Perforated-patch recordings under a series of voltage pulses. The cell was held at its resting potential (−47 mV) and membrane potential was jumped for 30 ms to depolarizing and hyperpolarizing values ranging from −120 to 30 mV at 10-mV increments. Bath solution contained 21 mM potassium. **B** Current/voltage (I/V) curve of K_{ir} channel activity in different $[K]_o$ concentrations. I/V relationship for the traces shown in **A**. Increasing $[K]_o$ from 7 mM to 21 mM shifted the inflection point toward the new equilibrium potential for K (E_K) and increased the conductance from 6.2 nS to 9.17 nS. **C** Pharmacological properties of the inward rectifier. I/V curve of K_{ir} channel activity at 21 mM $[K]_o$ before and after blocking of current by $BaCl_2$ 2 mM -▲- or CsCl 1 mM -●-

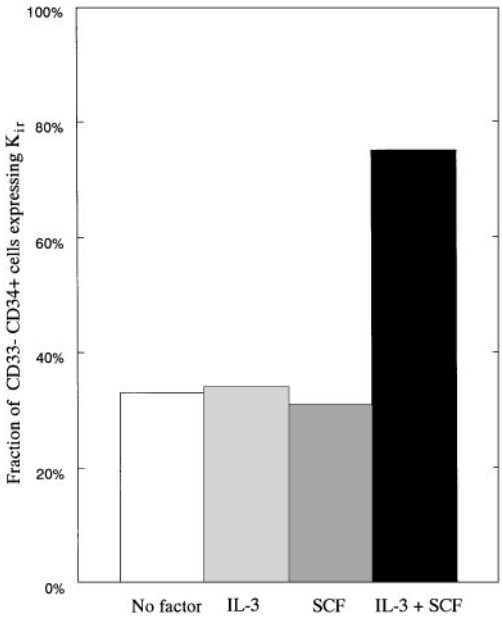

Fig. 2 Fraction of $CD33^-CD34^+$ cells expressing K_{ir} after 16 h of incubation with cytokines. 10/29 cells control, 9/27 with interleukin-3 (IL-3), 6/19 with stem cell factor (SCF), and 15/20 with both IL-3 and SCF. A total of 27 separate cord blood purifications were used each as a source for patching 1–2 cells per incubation group

$n = 6$, IL-3 + SCF 0.56 ± 0.15 nS/pF $n = 15$). The minimum K_{ir} current recorded in these cells (at −110 mV) was 35 pA and the overall mean K_{ir} current was 219 ± 39 pA. Of K_{ir}-positive cells, 40% expressed K_{ir} as the only K^+ current in the −100 to 0 mV range. The other 60% of K_{ir}-positive cells exhibited delayed rectifier K^+ currents detectable from −20 mV and above. These outward currents were unaffected by either 1 mM Cs^+ or 2 mM Ba^{2+}.

K_{ir} antisense oligodeoxynucleotide decreases channel RNA and inhibits channel expression

The recent cloning of several genes encoding K_{ir} [7], and in particular the mouse macrophage IRK1 [22], enabled the design of antisense oligodeoxynucleotides directed against the mRNA of this channel [21, 33]. Treating $CD33^-CD34^+$ cells with a K_{ir} antisense oligodeoxynucleotide (100 μg/ml) for 48 h in the presence of IL-3 and SCF significantly decreased the channel message with no effect on control S14 ribosomal protein message as detected by RT-PCR (Fig. 3). The effect of scrambled K_{ir} antisense-sequence oligonucleotides on the channel message was not statistically significant. The mean optical density (OD) ratios of K_{ir} DNA to S14 DNA bands, obtained from PCR cycle numbers 24, 28 and 32 are 1.06 ± 0.30 for control, 1.79 ± 0.46 for scrambled and 0.19 ± 0.01 for antisense-treated cells.

An F-test for significance between groups yielded $P = 0.001$ for antisense compared to control, $P = 0.017$ for scrambled compared to antisense, and $P = 0.31$ for scrambled compared to control. In accord with the low amount of K_{ir} mRNA detected by RT-PCR, treatment

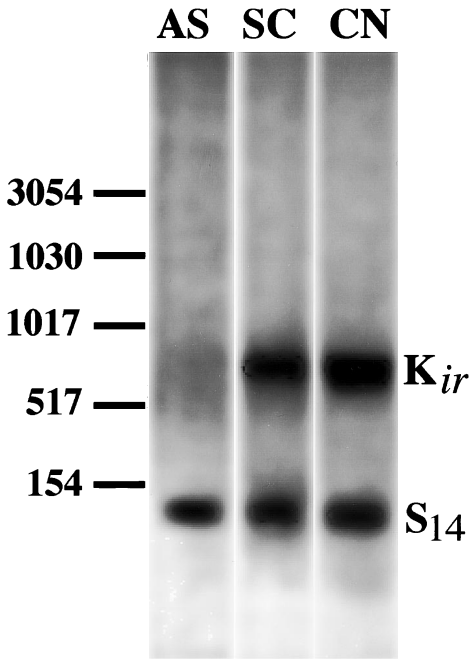

Fig. 3 Gel electrophoresis of RT-PCR products. $CD33^-CD34^+$ cells were incubated for 48 h in presence of K_{ir} antisense oligodeoxynucleotides (*AS*), scrambled oligodeoxynucleotides (*SC*), or without oligodeoxynucleotides (*CN*). Note the presence of S14 message in all cell groups while IRK1 message is absent in cells treated with antisense

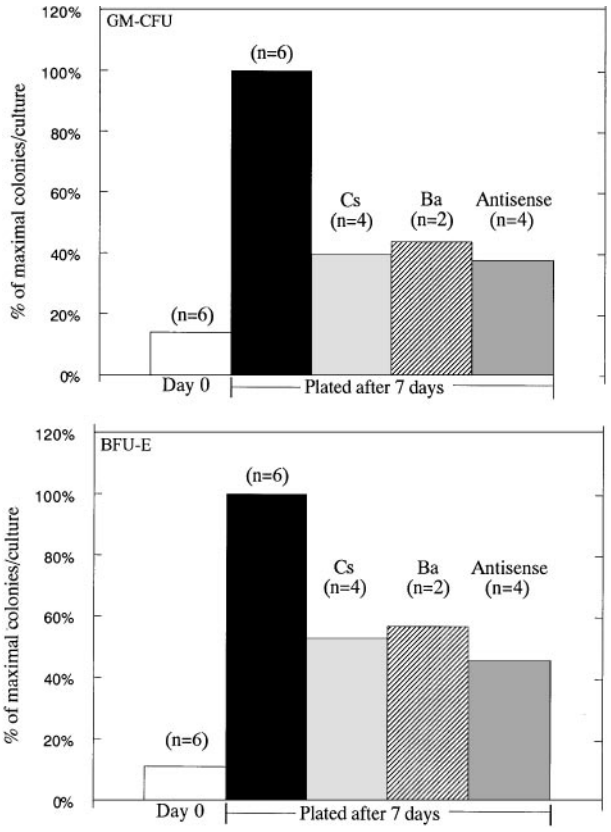

Fig. 4 Effect of K_{ir} blockers on the generation of hemopoietic progenitors from $CD33^-CD34^+$ cells in 7-day suspension cultures. *Top panel*, granulocyte-macrophage colonies; *bottom panel*, erythroid colonies. The histogram values for antisense are relative to scrambled oligodeoxynucleotide treated cultures

of $CD33^-CD34^+$ cells with the K_{ir} antisense for 48 h in the presence of IL-3 and SCF, also had an inhibitory effect on functional K_{ir} expression. Perforated patch-clamp recordings, under the same conditions, revealed functional expression of K_{ir} in only two of nine cells compared to seven of nine cells treated with the scrambled sequence oligodeoxynucleotides. The K_{ir} current values in these two cells were within the range of K_{ir} currents in cells not treated with antisense.

K_{ir} antisense oligodeoxynucleotide inhibits cytokine-induced progenitor cell expansion

Incubation of $CD33^-CD34^+$ cells in 7-day suspension cultures in the presence of IL-3 and SCF resulted in a 9 ± 1.4-fold enrichment of primitive erythroid (BFU-E) and a 7 ± 3-fold enrichment of granulocyte-macrophage (GM-CFU) progenitors, relative to the initial content of these progenitors at day 0 (80 ± 16/culture and 56 ± 22/culture, respectively; $n = 6$). Consequent to its inhibition of K_{ir} mRNA and K_{ir} expression, the K_{ir} antisense oligodeoxynucleotide inhibited generation of progenitors by $CD33^-CD34^+$ cells (Fig. 4). Erythroid and myeloid progenitor generation was inhibited to a similar extent. As shown in Fig. 4, 100% values (day 7) are 724 ± 255 (BFU-E) and 387 ± 48 (GM-CFU) per culture. Antisense group values are 231 ± 57 (BFU-E) and 108 ± 19 (GM-CFU). Scrambled sequence oligodeoxynucleotides also induced some expected [33] degree of inhibition of erythroid and myeloid progenitor generation. Scrambled oligodeoxynucleotide values are 456 ± 58 (BFU-E) and 263 ± 38 (GM-CFU).

While the scrambled oligodeoxynucleotide served as control for sequence-independent effects, an additional control for sequence-dependent side effects acting on targets other than K_{ir} was required [33]. To this end, proliferation of HL60 cells, which do not express K_{ir} constitutively [35] (confirmed under our culture conditions), was monitored and was found to be unaffected by 100 μg/ml of antisense oligodeoxynucleotide. MTT assay OD values following 48 h of culture were indistinguishable in the absence and presence of the oligodeoxynucleotide (1.30 ± 0.07 and 1.34 ± 0.03, respectively).

Effect of K_{ir} current blockers on cytokine-mediated expansion of hemopoietic progenitors

To test whether the essential function of K_{ir} for cell expansion is the actual K^+ current, we resorted to use

of the K_{ir} channel current blockers Ba^{2+} and Cs^{+}. We examined the effect of Cs^{+} (1 mM) or Ba^{2+} (2 mM), on cytokine-induced expansion of $CD34^{+}33^{-}$ cells during 7-day suspension cultures. These blockers were found to exert a similar inhibitory effect (40–60%) on progenitor cell generation, as K_{ir} antisense oligonucleotides. Absolute and relative values of inhibitory effects on progenitor production by the ion blockers Ba^{2+}, Cs^{+} and of antisense and scrambled oligonucleotides are compared in Fig. 4.

Discussion

The results presented here demonstrate the existence of an inwardly rectifying K channel in human primitive hemopoietic progenitor cells defined by membrane antigens $CD33^{-}CD34^{+}$. This channel is expressed in more cells following cytokine induction. When treated with channel blockers, both the current and cytokine-induced in vitro expansion into committed progenitors are inhibited. These findings strongly suggest that K_{ir} is essential for cytokine-induced growth and differentiation of primitive hemopoietic progenitors.

K_{ir} induction in $CD33^{-}CD34^{+}$ cells was only detected following concomitant exposure to both early-acting cytokines, IL-3 and SCF. This is remarkable in view of the synergistic effect of both cytokines on the generation of myeloid and erythroid progenitors by these cytokines [2, 28]. The molecular mechanisms underlying this synergism are presently unclear. While the SCF receptor (c-kit) belongs to the platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) family of tyrosine kinase receptors, the receptor for IL-3 is devoid of endogenous tyrosine kinase activity [29]. Although differing in endogenous catalytic function, ligand binding to both receptors induces rapid and transient tyrosine phosphorylation of the receptors as well as of various cellular substrate proteins [29, 30]. Signal transduction studies in a human (MO-7) leukemic cell line which responds to both IL-3 and SCF, have shown that both regulators induce similar phosphorylation of some (p42MAPK, p72Raf-1 kinase and JAK2 kinase), but not all (i.e. PLC-gamma, PI-3 kinase) cellular substrate proteins [3, 20, 30]. Additional studies suggesting that SCF and IL-3 may share some common intracellular signaling pathways have shown that both cytokines exert a synergistic effect on the expression of immediate-early genes such as c-*fos*, *jun*-B, c-*myc* and *egr*-1 [16]. These observations point to two possible mechanisms by which cytokine-dependent K_{ir} expression can occur, namely by K_{ir} activation and K_{ir} induction. The first is consistent with reports on tyrosine kinase modulation of latent ion channels as described for other K channels [18], while the latter involves transcription as documented in the case of K channel induction by Ras/Raf [19]. Since K_{ir} was not seen in perforated-patch recordings of $CD33^{-}CD34^{+}$ cells up to 30 min after stimulation with IL-3 and SCF, it seems more likely that an induction mechanism, rather than mere activation, is involved in expressing this channel.

The incomplete inhibition of in vitro generation of hemopoietic progenitors by antisense treatment may be attributable, among other mechanisms, to either low efficacy with only a partial blocking effect on mRNA translation, or to a slow K_{ir} ion channel turnover whereby cells already possessing K_{ir} are unaffected. In contrast to the delayed action of an antisense oligonucleotide treatment, Cs^{+} and Ba^{2+} block K_{ir} immediately and efficiently, nevertheless some 40% of all colony-forming cells were generated. This suggests that the incomplete inhibition of progenitor cell expansion may be due to either cellular compensatory mechanisms or to some of the $CD33^{-}CD34^{+}$ cells having already undergone K_{ir}-dependent differentiation. The fact that 30% of control unstimulated $CD33^{-}CD34^{+}$ cells express K_{ir} is in accord with the notion that they comprise a heterogeneous population of cells with respect to their developmental status and hemopoietic capacity.

The similar inhibition of progenitor cell expansion by K_{ir} antisense and K_{ir} current blockers, indicates that indeed the K^{+} current flow through K_{ir} is essential and not mere expression of K_{ir} as a protein. Myeloid and erythroid progenitor generation was inhibited to a similar extent by all K_{ir} inhibitors (Fig. 4). This suggests that K_{ir} is essential for stages prior to lineage determination and is not restricted to any specific hemopoietic pathway. The degree of identity of the human $CD33^{-}CD34^{+}$ progenitor cell K_{ir} to other inward rectifier channel sequences is being assessed currently by subcloning and sequencing PCR-derived products.

While the fraction of $CD33^{-}CD34^{+}$ cells expressing K_{ir} was increased following stimulation with SCF and IL-3, the cells which were negative for K_{ir} may carry K_{ir} below the whole-cell recording detection levels. Preliminary single-channel analyses revealed 60- to 70-pS channels having K_{ir} characteristics in cell-attached recordings. Taken together with a mean K_{ir} current of -219 pA at -110 mV, measured in perforated patches, the number of channels is in the range of 100 per cell. Normalized to cell surface area, based on capacitance measurements, the channel density is about 1 channel per 6 μm^2. Although only a few molecules would go undetected in the perforated-patch recording at our signal to noise ratio (see Materials and methods), this may be sufficient to subserve cellular K_{ir} functions, considering the extremely high throughput and efficiency of ion channels [14]. It will be interesting to test whether K_{ir} is not only induced but also modulated [26] by any of the second messengers activated by IL-3 and SCF

during progenitor cell expansion, allowing for a transient recruitment of K_{ir} during an essential point in time.

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