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Bone marrow stroma inhibits proliferation and apoptosis in leukemic cells through gap junction-mediated cell communication

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Abstract

Normal and leukemic blood cell progenitors depend upon the bone marrow (BM) stroma with which they communicate through soluble and membrane-anchored mediators, adhesive interactions and gap junctions (GJ). Regarding hematopoiesis, it is believed that it can be influenced by connexin expression, but the exact role of GJ in cell death and proliferation is not clear. Using flow cytometry, we monitored the division rate of leukemic cell lines, communicating and not communicating with stromal cell line through GJ. We found that GJ-coupled cells (i) did not proliferate; (ii) were kept in G0; and (iii) were protected from drug-induced apoptosis when compared to either total or uncoupled cell population. We conclude that GJ coupling between stroma and leukemic lymphoblasts prevents proliferation, keeping cells in a quiescent state, thus increasing their resistance to antimitotic drugs. Since GJ are particularly abundant in the sub-endosteal environment, which harbors blood stem cells, we also asked which cells within the normal human BM communicate with the stroma. Using a primary BM stroma cell culture, our results show that 80% of CD34+ progenitors communicate through GJ. We propose that blood cell progenitors might be retained in the low-cycling state by GJ-mediated communication with the hematopoietic stroma.
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Keywords: gap junctions; connexin; leukemia; stem cell; proliferation; apoptosis

Abbreviations: 7AAD, 7-aminoactinomycin D; AO, acridine orange; BM, bone marrow; CBX, carbenoxolone; Cx, connexin; GJ, gap junction; GJIC, gap junction intercellular communication; Da, Daltons; MTX, methotrexate

Introduction

In adult mammals, bone marrow (BM) is the major site of hematopoiesis. The BM is composed of hematopoietic and stromal cells, blood vessels, nerves and extracellular matrix proteins.[1,2] The cellular components interact through intercellular communications, cytokines and hormones,[3] which together determine the rate of blood cell production and renewal. The result of all these interactions is the adequacy of hematopoiesis for the systemic needs, specifically regulating the number of cells in each compartment.

The interactions between hematopoietic and stromal cells are also important for the maintenance of the undifferentiated pluripotent and long-lived stem cell.[3–5] Besides communication via surface ligands and their corresponding receptors, cells can communicate through gap junctions (GJs): specialized membrane structures that bridge the cytoplasms of the interacting cells. They are formed by connexins (Cx's), which are proteins inserted into the plasma membrane as a hemichannel or connexon.[6] A complete gap junction pore is formed by the association of two hemichannels from two communicating cells.[7] This pore is permissive for the exchange of water, inorganic ions, small metabolites and secondary messengers, allowing functional integration of continuous cell systems. Early ultrastructural and subsequent cytochemical studies of the BM hematopoietic environment had described GJs in the stroma and blood vessels.[8–11] Transfer of Lucifer Yellow (a GJ probe) among stromal cells and between stromal and blood cells had shown the existence of functional GJ-mediated cell interactions.[12,13] This was further confirmed by the demonstration of electric cell coupling between purified immature progenitors and a stromal cell line.[14]

Association of increased Cx expression and increased blood cell production has raised the hypothesis that Cx's could participate in the control of hematopoiesis.[11,15,16] This hypothesis was subsequently confirmed in studies on mice with targeted disruption of the gene encoding Cx43.[17] These animals presented a deficient capacity to regenerate blood cell production after cytoablative treatments. Moreover, using radiation chimeras, the authors showed that impaired T- and B-lymphocyte maturation was due to a defect in the stromal cells of both the thymus and BM. This confirms Rosendaal hypothesis[11,15,18] that Cx43 expression in the stroma is critical during early growth and regeneration of the hematopoietic system, but not for maintenance of the steady-state blood cell production.

Similar to normal hematopoiesis, proliferation of leukemic cells is also largely dependent upon the BM environment.[19,20] In leukemia, a small number of autorenewable clonogenic cells, leukemic stem cells,[21,22] depend upon the stroma to survive.[23–25] Their quantification reflects disease malignancy and predicts treatment outcome.[20] Several studies had clearly shown that leukemic cell adhesion to stroma, which involves adhesion molecules as integrins and CD44, or juxtacrine interactions, as those mediated by c-Kit, supports leukemic lymphoblasts survival and protects them from apoptosis.[4,24]

In the present study, we have addressed the question of whether GJ-mediated intercellular communications (GJIC) between the stroma and leukemic cells can control cell growth and death. We have established an *in vitro* model using the S17 murine bone marrow stroma cell line and some leukemic lines. Similar to other murine cell lines, S17 cells sustain long-term *ex vivo* growth of human blood cell progenitors.[26–29] By monitoring the calcein dye transfer from the stroma to leukemic cells we could describe, using flow cytometry, the effect of GJIC on leukemic cell growth and death. Moreover, using human BM, we address which hematopoietic cells communicate through GJ when plated over primary stromal cultures.

Results

Morphology of stromal–leukemic cell cocultures, and identification of GJ-mediated cell communication

Morphological analysis of stromal–leukemic cell line cocultures showed similarity to primary long-term BM cultures.[30] Leukemic cells infiltrated the pre-established stroma, forming typical cobblestone areas (Figure 1a), which are representative of primitive hematopoietic progenitor-cell niches *in vitro*.[31] Ultrastructural analysis showed close cell membrane contacts, suggesting juxtacrine interactions (Figure 1b and c). In order to check if GJs could take place between the stroma and the leukemic cell line studied, we looked for the presence of message for various Cx's in both cell lines. Indeed, as shown in Figure 1d and e, both S17 and CCRF-CEM lymphoblasts express mRNA for several connexins. Regarding protein expression, several studies had shown the importance of Cx43 for the establishment of GJ between BM cells.[12–15,17,32] Using immunofluorescence, we also detected Cx43 in a typical punctuate distribution on both cell membranes (data not shown). Functional GJ–mediated communications in S17 cell layers were also confirmed by intercellular transference of Lucifer Yellow, a 457 Da dye permeable only through GJs (data not shown).

We monitored the presence of GJ-mediated cell interactions between leukemic and stromal cell lines using calcein loading-transfer assay. Calcein is a 623 Da fluorescent dye, permeable only through GJ, which can be read on the flow cytometer, enabling functional studies of coupled and uncoupled cell population, with minimum manipulation. Calcein was loaded into the S17 stroma, which was cocultured with leukemic blasts for 3 days, and analyzed by flow cytometry. Owing to the great difference of light dispersion, S17 stroma and leukemic cells can be easily distinguished by forward and side scatter, as shown in Figure 2. Transfer of dye between stroma and leukemic cells was readily detectable after 72 h of coculture, when 20% of leukemic blasts were labeled with calcein (Figure 2b). Contamination of labeled stromal cells in the leukemic gate was discarded since, at day 0, no fluorescence was detected in the assigned gate (Figure 2a), while cells in the stroma gate showed extremely high levels of fluorescence labeling.

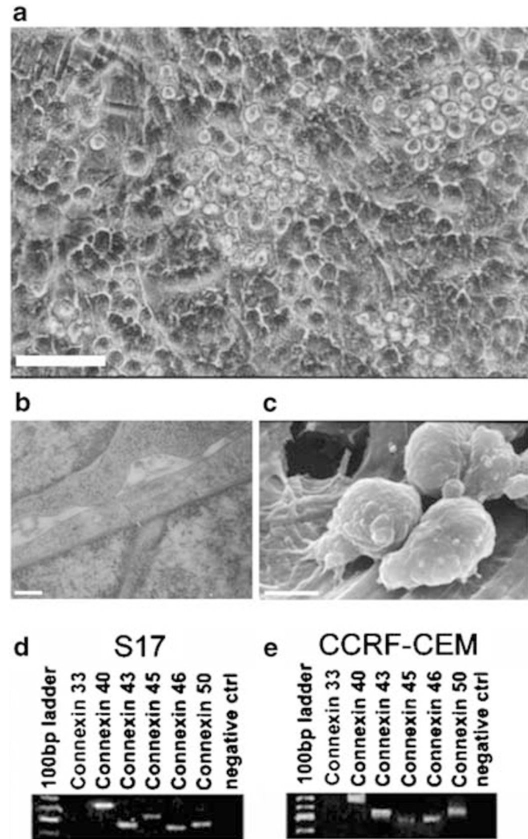

Figure 1 Morphological aspects of interaction between CCRF-CEM leukemia cell line and S17 stroma cell line. (**a**) S17 and CCRF-CEM cell aspect at phase contrast microscopy. Infiltrating lymphoblasts can be identified by lack of light transmission forming typical cobblestone areas. Noninfiltrating blasts are seen growing over the stroma as bright cells (bar=21 μm). (**b**) S17/CCRF-CEM coculture at transmission electron microscopy(bar=0.25 μm). (**c**) S17/CCRF-CEM coculture aspect at scanning electron microscopy. Leukemic cells show extensive membrane processes over the stroma cell (bar=5 μm). (**d**) mRNA Cx expression in murine S17 cell line and (**e**) in human CCRF-CEM lymphoblast cell line. Amplicons size variability between (D and E) are due to species-specific differences in Cx genes

Thus, the above results indicate the presence of functional GJ between leukemic and stromal cell lines.

GJIC increases the number of cells in the G0 phase of the cell cycle and inhibits cell proliferation

Cell cycle kinetics was monitored in order to study the influence of stroma-dependent cell interactions on leukemic cell proliferation.

Acridine orange (AO), which stains DNA and RNA in different colors, was used in order to distinguish cells in G0,

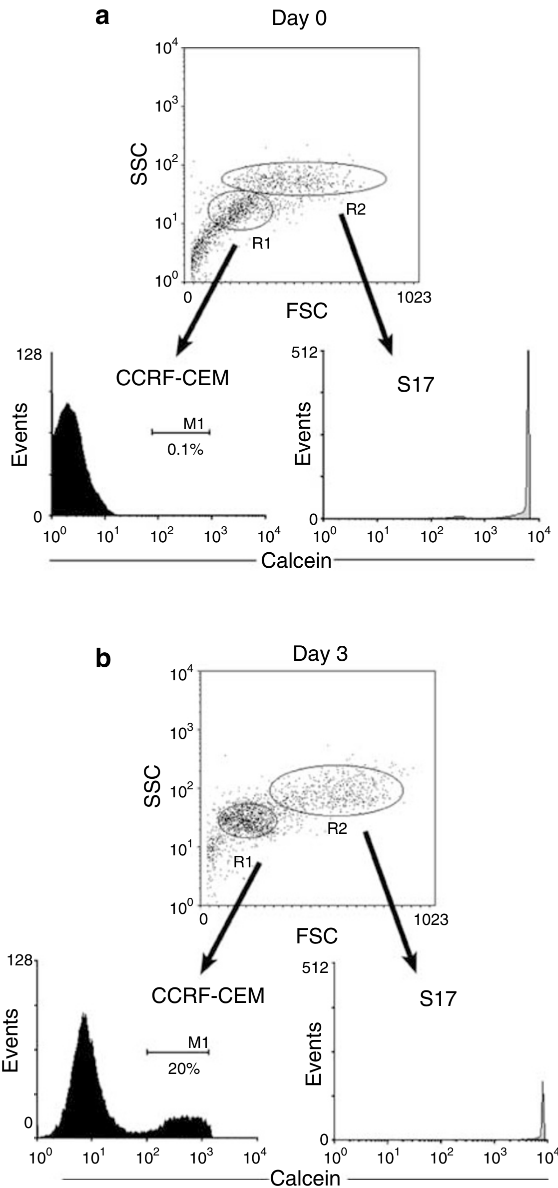

Figure 2 Flow cytometry strategy for calcein loading-transfer assay to measure GJIC between stroma cells and leukemic cells after 3-day coculture. S17 cells loaded with calcein were cocultured for 0- (**a**) and 3- (**b**) day period with CCRF-CEM cells. Leukemic cells were analyzed for calcein fluorescence under the leukemic gate (**a** and **b**, lower left), while S17 used the stromal gate (**a** and **b**, lower right). Note the low background level of fluorescence observed in the CCRF-CEM gate at day 0 (**a**, lower left). At day 3, GJ-mediated communication was quantified within the leukemic cell population (**b**, lower left). This experiment is representative of four independent assays

G1 and S/G2/M phases of the cell cycle. Figure 3 shows that in stroma-free cultures (white bars), almost all the CCRF-CEM cells are committed to the cell cycle, with 49% of the cells in G1 and 47% in S/G2/M. However, when cultured with S17 cells, commitment with the cell cycle diminishes, and the number of cells in S/G2/M drops to 28% (light gray bars). Even more striking is the increase from 3%, in stroma-free cultures, to 20% in the number of cells in G0 in the cocultures (light gray bars). With respect to cell proliferation, these results indicate

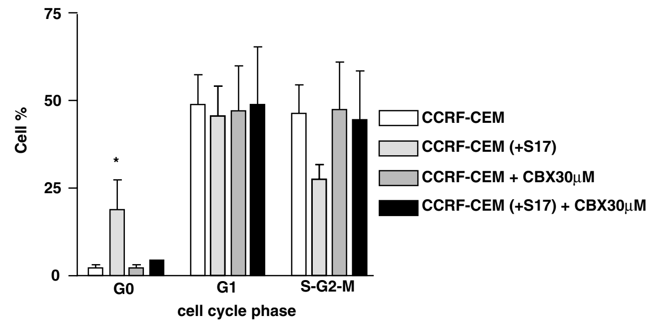

Figure 3 Cell cycle analysis of CCRF-CEM cells grown either in the presence or absence of S17 stroma. AO was used to distinguish G0, G1 and S/G2/M as explained in Material and Methods. Leukemic cells were cultured alone (white and dark gray bars) and in the presence of stroma cells (light gray and black bars) without (white and light gray bars) and with CBX (dark gray and black bars). Percentages of cells in G0, G1 and S/G2/M phases of the cell cycle are indicated. Mean of four experiments. *means $P<0.05$ *t*-paired test

that the stroma has an inhibitory effect, which cannot be attributed to an arrest in G1, but rather to an effect that retains the cells in a quiescent state (G0).

To investigate the role of GJs over the cell cycle, we used carbenoxolone (CBX), a drug that inhibits GJIC[33,34] in the cocultures of CCRF-CEM with S17 and deserved the different phases of the cell cycle using AO as above (Figure 3 – black bars). Carbenoxolone abrogated the increase in the number of cells in the G0 phase of the cycle, showing the modulation of leukemic cell cycle by GJ.

The use of calcein loading-transfer assay led us to monitor directly the effect of GJIC on leukemic cell proliferation. Using calcein and PKH 26 red dye, we monitored the proliferation of leukemic cells, cultured alone and in the presence of S17 cell line. Leukemic cells that established a functional gap junction with stroma (calcein positive) were distinguished from those that did not (calcein negative) as shown in Figure 2. When compared to control cultures, CCRF-CEM/S17 cocultures carried out in trans-wells as well as calcein-negative cells, showed little cell proliferation changes (Figure 4a). Strikingly, whenever GJIC was established, calcein-positive cells had an intense growth inhibition (Figure 4a). To verify if the inhibition observed above was exclusive to CCRF-CEM, we additionally tested two acute myeloid leukemias, HL60 and KG1. Both cell lines can differentiate *in vitro* into different lineages, indicating some pluripotentiality.[35–37] Confirming the results observed with communicating CCRF-CEM, both HL60- and KG1 calcein-positive cells have their division index close to zero (Figure 4b and c). It is important to emphasize that calcein *per se* does not interfere with leukemic cell proliferation (Figure 4a, inset). Thus, the above results indicate that stroma–leukemia GJs inhibit cell proliferation, with the induction of a quiescent nonproliferative state in the leukemic cells.

GJ inhibits drug-induced cell death

Cell population kinetics depends upon the rate of cell growth and death. We found a correlation between cell proliferation inhibition mediated by GJs and the presence of cells in the G0 phase of the cell cycle described above. However, the role of

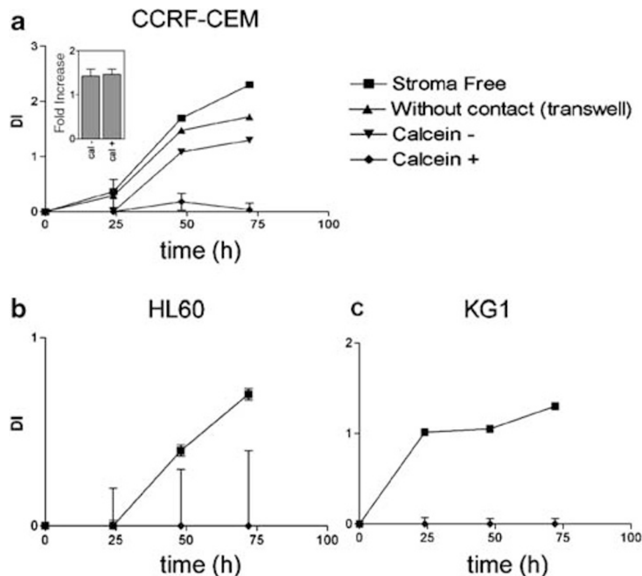

Figure 4 GJ-mediated intercellular communication with S17 inhibits proliferation of leukemia cells. Division index of CCRF-CEM cells (**a**) growing without (control) and with stroma, either coupled (cal +) or not (cal−) and without stroma contact (noncontact) was calculated based on PKH26 red (FL2) decay as described in Material and Methods. Note that calcein does not alter CCRF-CEM proliferation (inset). Proliferation pattern of HL60 (**b**) and KG1 (**c**) cells cultured in the absence of stroma and with stroma. The results for stroma-free cells and cal + are shown. Bars indicate 95% confidence interval. Representative of three experiments

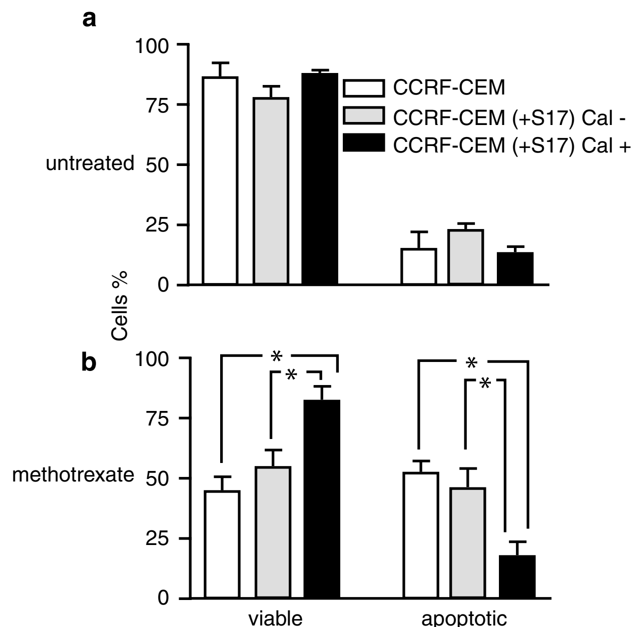

Figure 5 Influence of GJ-mediated intercellular communication of stroma and leukemic cells over life and death of leukemic blast. Cells were cultivated as for standard calcein loading-transfer assay (72 h) and analyzed with 7AAD for apoptosis quantification. Untreated (**a**) and MTX (1 μM for the last 24 h) treated cultures (**b**). Relative numbers of viable and apoptotic cells are indicated. Stroma-free cultures (white bars), nonstroma-coupled leukemia (gray bars) and GJ stroma-coupled leukemic cells (black bars). *means $P<0.05$ t-paired test

GJIC between stroma and leukemic cells in cell death is still undetermined. We have addressed this issue by associating calcein loading-transfer assay with 7-amino actinomycin (7AAD) staining, monitoring viability and apoptotic cell death. CCRF-CEM cells have some degree of spontaneous apoptosis when grown in the absence of S17. When in coculture with S17 cells, viability and spontaneous apoptosis of calcein-negative leukemic cells was not different from those of control cultures (Figure 5a – gray and white bars, respectively). However, coupled cells showed a nonsignificant, but a consistently small increase in the percentages of viable cells. Conversely, there was a small decrease in the percentages of apoptotic cells (Figure 5a – black bars).

Stromal cells are believed to be responsible not only for the survival of leukemic cells in pathological conditions but also during chemotherapeutic treatments.[38] In order to test whether GJs could protect leukemic cells from drug-induced apoptosis, cell cultures were treated for 24 h with methotrexate (MTX). This drug inhibits dihydrofolate reductase, an enzyme critical for DNA synthesis and cell proliferation. Under these conditions, leukemic cell viability fell below 50%, while apoptosis increased over 50% (Figure 5b – white bars). When leukemic cells were maintained over a calcein-loaded S17 stroma, calcein-positive cells viability increased up to 80%, while apoptosis dropped below 25%, representing a protection above 50% (Figure 5b – black bars) when compared with MTX-treated noncoupled cells (Figure 5b – gray bars).

Taken together, these results indicate that GJIC modulate both leukemic cell proliferation and death.

CD34 + progenitor cells communicate through GJ with human stromal cells in primary cultures

A stem cell's major characteristic is its ability to self-renew and perpetuate its progeny. This ability is frequently related to a low proliferation rate that keeps cells quiescent. Adding to this, our results point to the role of GJ in the cell cycle, inhibiting proliferation and keeping cells in a quiescent state. On the other hand, GJ is known to be particularly abundant in the sub-endosteal environment that harbors blood stem cells.[10,11,39] Altogether, we propose that if GJ is important to keep the stem cells quiescent within BM hematopoietic cells, $CD34^+$ cells should be able to establish GJIC with stromal cells.

To answer this question, a human primary stromal culture was established, loaded with calcein and cocultured with whole human BM cells. After a 3-day culture, cells were stained with anti-CD34PE and anti-CD45 PercP mAb and analyzed in the flow cytometer for the presence of calcein. Figure 6 shows that 80% of CD34 + cells do communicate through GJ with the stroma, while only 15% of CD34− are calcein positive, suggesting that GJ might play a role in the maintenance of normal stem cells.

Discussion

GJs can integrate electrically or metabolically large tissue sectors and delimited cell clusters. In BM, Cx43 is expressed on bone-lining endosteal and reticular stromal cells that are in direct contact with hematopoietic progenitors and on endothelial cells.[10,11] This BM region was recognized to be a

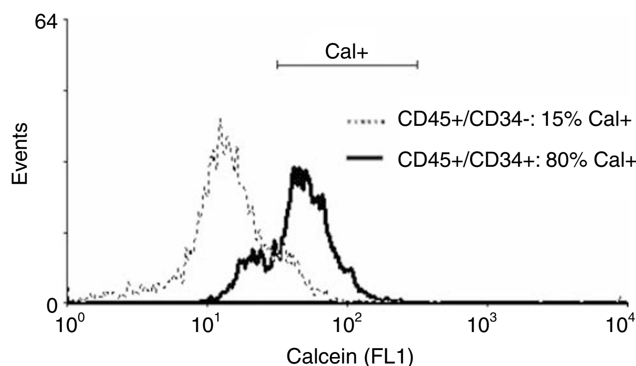

Figure 6 Functional GJ analysis of *ex vivo* primary bone marrow cells. Primary hematopoietic bone marrow cells were cocultured with primary stroma cells. Primary stroma cells were loaded with calcein and after 3-day culture, all cells were detached and stained for hematopoietic stem/progenitor cells (CD45 and CD34). Histograms show calcein staining for CD45+/CD34+ and CD45+/CD34− gated populations. Most hematopoietic early progenitor/stem cells (80% of CD45+/CD34+) are cal+, indicating functional coupling via GJs with primary stroma

niche for homing of stem and early hematopoietic progenitors.[40] Also, Cx43 GJ are upregulated during development and after cytoablative treatment.[15] In Cx43 knockout mice, the number of hematopoietic stem cells (Sca-1$^+$/Lin$^-$) was clearly reduced,[17] and male germ stem cells were significantly reduced.[41] Corroborating for the involvement of GJ in stem cell regulation, purified human immature CD34$^+$ progenitors had been shown to communicate with a stromal cell line through Cx43 GJs.[14] In fact, in bulk BM hematopoietic population, 80% of the more immature CD34$^+$CD45$^+$ cells do connect to the stroma through GJ, while the more mature pool does not (Figure 6), suggesting a possible role for this interaction in the maintenance of the stem cell pool.

The fate of a stem cell, malignant or not, is to maintain itself and perpetuate its progeny.[42] This property has been suggested to be associated with quiescence, while engaging in cycling has been related to differentiation.[43] The mechanisms maintaining the 'stemness' are unknown, but certainly involve cell cycle arrest. Moreover, GJ has long been implicated in malignant cell development and differentiation.[44,45]

With our results, we further suggest that GJ is at least one of the mediators of cell cycle regulation. We show that leukemic cells communicating with the stroma do not proliferate (Figure 4). Cell cycle studies demonstrate that these leukemic cells are arrested in G0, since treatment with CBX, an uncoupling agent, inhibits the increase in the number of cells in G0 (Figure 3) when in coculture with stroma.

Through an indirect assay, GJs have been proposed to make hematopoietic stem cells escape stromal inhibition[46,47] and to engage in proliferation, commitment and production of blood cells.[18] In contrast, using a direct assay, we have shown that GJs are actually involved in the maintenance of quiescence. We also suggest that such regulation can be achieved through signaling for asymmetric cell division. GJs are permeable to second messengers, such as cAMP, which can polarize asymmetric cell divisions.[48] Thus, the reduced amount of stem cells in the murine Cx43-KO phenotype[17,41] could be interpreted as the loss of asymmetry resulting in proliferation and differentiation with loss of 'stemness'. Besides elimination of cell growth (Figure 4), our results show nearly 50% inhibition of drug-induced apoptosis in leukemic cells that communicate with the stroma (Figure 5). This inhibition of drug-induced apoptosis is probably due to an arrest in the G0 phase of the cell cycle, which was dependent upon the GJIC (Figure 3). Resistance to drug-induced apoptosis may explain the quiescent carcinoma micrometastasis and residual disease observed in the BM, which are associated with poor prognosis.[20]

Cell proliferation and programmed cell death are opposed in tissue homeostasis, but each one depends in part upon rather similar cell mechanisms: many molecules involved in signal transduction leading to cell proliferation are shared with common death signal pathways.[49,50] This gives rise to the idea that cell cycle and apoptosis are not opposing events and can thus be regulated by the same mediators.

Altogether, the data presented herein can add to the understanding of the mechanisms that contribute to the maintenance of normal and malignant stem cells. We are currently investigating the role of GJ in micrometastasis and normal stem cell maintenance.

Materials and Methods

Cell cultures

Cell lines were obtained from the Rio de Janeiro Cell Bank (BCRJ, Federal University of Rio de Janeiro, Brazil). Murine S17 BM stroma cells were used according to authorization by Dr K Dorshkind.[27] Routinely, cells were maintained in the RPMI-1640 medium (Sigma, St Louis, MO, USA) supplemented with10% fetal bovine serum (FBS – HyClone, Logan, UT, USA), 2.2 g/l $NaHCO_3$ and 2 g/l HEPES (pH 7.4), at 37°C under 5% CO_2. All the experiments were carried out using S17 cells below the 29th passage of the original stock. All leukemic cells were maintained in the same medium, with cell density kept at 10^5–10^6 cells/ml. Cocultures consisted of leukemic cells (10^5/ml) plated over semiconfluent layers of S17 cells. When indicated, the leukemic CCRF-CEM cells were cultured in the upper compartment of trans-well culture plates (0.45 μm – Nunc, Roskilde, Denmark), with S17 cells in the lower compartment.

Normal primary BM samples were obtained from healthy BM transplantation donors under informed consent. Primary BM stroma were generated by cultivating Ficoll–Hypaque-treated BM (Histopaque™, Sigma) in McCoy 5A medium supplemented with 10% v/v FBS (HyClone) and 2 mM L-Glu (Sigma), namely M10. Cultures were plated in 75 cm^2 cell culture vessels for over 2 weeks before the first passage. Primary BM stroma cultures were used between second and fourth passages. Coculture of normal BM stem/progenitor cells with primary BM stroma was performed using mononuclear cells from BM, after a 24 h period of adhesion in M10 media. After this, cells were harvested, counted and plated (10^6/ml) over confluent PBMS.

Cell viability was evaluated before every plating using trypan blue exclusion assay. Viability was always superior to 95% at the beginning of each experiment.

RT-PCR

S17 or CCRF-CEM cells were collected and treated with 1-ml Trizol™ (Gibco-BRL/Life Technologies Inc., Grand Island, NY, USA), followed by

Table 1 Cx primers

Cx	Sense	Antisense
Cx37	GAACATCAGATGGCCAAGAT	GGATCATAAACAGTGGAACT
Cx40	GCACACTGTGCGCATGCAGG	CTGCTGGCCTTACTAAGGCG
Cx43	ATCCAGTGGTACATCTATGG	CTGCTGGCTCTGCTGGAAGG
Cx45	TGGAGCATGGTGAGGCAGAC	CTTAGCATTGGACAGCTCAG
Cx46	GGAACCAATGCGTACAGGGA	CCTCTGCCTTGCGCATCGTT
Cx50	CGCATGGAGGAGAAGCGCAA	GGGCTGGTCTCCACCAT

PCR cycles were: 96°C 3 min (denaturation), 33 cycles with 30 s at 96°C, 30 s at 55°C (annealing) and 60 s at 72°C (polymerization). Samples were submitted to 72°C for 10 min for final polymerization extension. Thermal cycle was performed in an automated programmed thermal cycler (Perkin-Elmer)

total RNA extraction according to the manufacturer's protocols. First-strand cDNA synthesis was carried out using 1 μg RNA, Poli-dT primers and Superscript II (Gibco-BRL) according to the manufacturer's instructions. Primers sequence and PCR reaction parameters were performed as described in Table 1.

Amplicons were analyzed on standard agarose electrophoresis gel, using ethidium bromide and visualized by UV-transilluminator apparatus, and registered with Eagle Eye (Stratagene, La Jolla, USA) digital gel acquisition system.

Flow cytometry

Stem/progenitor cell staining

Normal bone marrow cultures and cocultures were suspended with EDTA (Sigma) solution (0.05% in phosphate-buffered saline, PBS) for 10 min at 37°C. Cells were washed with PBS containing 2% of normal human AB serum, incubated with anti-CD45-PERCP (Pharmingen) and anti-CD34-PE (Pharmingen), for 20 min, washed and fixed.

The flow cytometry studies were carried out using a FACSCAN® cytometer with a CellQuest® (Beckton Dickinson, San José, CA, USA) support software, and analyzed with WINMDI® PC application.

Electron microscopy

S17 cells were plated in 24-well tissue culture plates on top of polystyrene coverslips (Nunc, Roskilde, Denmark), and grown to confluence. 1×10^3 CCRF-CEM cells/ml were plated over the S17 monolayer and cultured for 72 h. Coverslips with stroma and leukemic cells were fixed with glutharaldehyde and routinely processed for transmission and scanning electron microscopy (TEM and SEM) as previously described.[51]

Calcein transfer assay for GJ-mediated intercellular communication

GJ-mediated intercellular communications between the stroma and leukemic cells were monitored by flow cytometry, following the protocol described by Czyz *et al*.[52] with slight modifications. Briefly, stroma cells were grown to semiconfluence (5×10^4 cells/cm^2) in six- 12-or 24-well plates. They were washed with 0.9% NaCl solution followed by an RPMI-1640 culture medium. Stroma monolayers were loaded with 1 μM calcein AM (Molecular Probes, Eugene, OR, USA) for 2 h, and extensively washed with a serum-free followed by serum-supplemented medium. After removal of the extracellular calcein, leukemic cells were added in 1 : 1 ratio in the latter medium. Control cultures of leukemic and stroma cells were prepared simultaneously. After 72 h, cells were trypsinized and washed with PBS supplemented with 5% FBS, fixed in 4% paraformaldehyde in PBS and analyzed by flow cytometry.

When indicated, CBX (Sigma, St Louis, MO, USA) was used in the coculture system to inhibit GJ-mediated cell communication. Control assays indicated that 30 μM CBX was devoid of cytotoxic effect for stroma and leukemic cells.

Cell cycle analysis

In order to dissociate G0 and G1 cell cycle phases of leukemic cells maintained with or without the S17 stroma, DNA and RNA content were measured using AO.[53] Briefly, leukemic and stroma cells were harvested as described, and cell suspensions were adjusted to 2×10^6 cells/ml with PBS containing 5% FBS. Subsequently, 200 μl was transferred to flow cytometer tubes and supplemented with 500 μl denaturing solution (0.3% saponin, 0.08 N HCl and 0.15 N NaCl) and incubated for 15 min on ice, followed by 1.5 ml staining solution (citrate-phosphate buffer, pH 6.0, 0.15 N NaCl, 1 mM EDTA-Na_4 and 10 μg/ml AO). The resulting suspension was analyzed by flow cytometry within 15 min. For acquisition, double discrimination mode was used with FL1. Data were plotted for analysis as FL1A (for DNA) $\times$ FL3H in linear scale (for RNA). Cells in G0 are discriminated as they show the same amount of DNA (FL1A) as cells in G1, with less RNA (lower FL3H).

PKH 26 cell labeling and cell proliferation

Leukemic cell proliferation was monitored with or without the underlying stroma (control) using the PHK26-GL red dye (Sigma, St Louis, MO, USA) as previously described.[54]

Cocultures were carried out as described above. At the indicated time points, the cells were harvested and the data acquired on the flow cytometer. For analyses, CCRF-CEM cells were gated based on SSC/FSC parameters and FL1 staining (cal+ or cal−) and DI was calculated according to FL2 fluorescence decay as proposed by Ashley *et al*.[54] Accordingly

$$2^{\mathrm{DI}} = F_0/F_{\mathrm{T}}$$

As though

$$\log 2^{\mathrm{DI}} = (\log F_0/F_{\mathrm{T}})$$

$$\mathrm{DI} \log 2 = (\log F_0/F_{\mathrm{T}})$$

$$\mathrm{DI} = (\log F_0/F_{\mathrm{T}})/\log 2$$

where DI is the division index, F_0 is the geometric mean of fluorescence at time 0, and F_{T} is the geometric mean of fluorescence at time T.

The 95% confidence interval (CI 95%) of DI was calculated by

$$\mathrm{DI} \pm 2\sqrt{p\mathrm{EPF}_0^2 + p\mathrm{EPF}_{\mathrm{T}}^2}$$

Apoptosis

The (7AAD Molecular Probes) staining was used as a simple and accurate method for detecting apoptosis. 7AAD was dissolved in DMSO (Sigma) at 10 mg/ml stock solution. A working solution was prepared by diluting it to 200 μg/ml and kept at -20°C protected from light. Staining was performed as described by Philpott *et al.*[55] with minor modifications. Briefly, leukemic cells cultured in the presence or absence of calcein-loaded stroma were harvested, and washed in PBS containing 5% FBS. A volume of 20 μl of 7AAD (200 μg/ml) was added to the final pellet, and incubated for 30 min, protected from light. Cells were subsequently washed with PBS containing 5% FBS, fixed with 4% paraformaldehyde in PBS and analyzed in a flow cytometer within 30 min. Viable, early or late apoptotic cells were identified according to the red fluorescence intensity (FL3) after gating, either calcein-positive or -negative leukemic cells.

For the study of MTX-induced apoptosis, leukemic cells cultured alone or with S17 stroma were grown for 48 h. MTX was subsequently added to the culture medium at a final concentration of 1 μM, and apoptosis was monitored after 24 h.

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