Brief Report

LYMPHOID NEOPLASIA

Stromal cell–mediated glycolytic switch in CLL cells involves Notch-c-Myc signaling

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Key Points

- Stromal cells promote a glycolytic switch in CLL cells in a Notch-c-Myc signaling-dependent manner.
- Targeting glucose metabolism or the Notch-c-Myc signaling pathway could be exploited to breach stromal cell–mediated CLL drug resistance.

It is well established that the stromal niche exerts a protective effect on chronic lymphocytic leukemia (CLL) cells, thereby also affecting their drug sensitivity. One hallmark of malignant cells is metabolic reprogramming, which is mostly represented by a glycolytic shift known as the Warburg effect. Because treatment resistance can be linked to metabolic alterations, we investigated whether bone marrow stromal cells impact the bioenergetics of primary CLL cells. In fact, stromal contact led to an increase of aerobic glycolysis and the cells' overall glycolytic capacity accompanied by an increased glucose uptake, expression of glucose transporter, and glycolytic enzymes. Activation of Notch signaling and of its direct transcriptional target c-Myc contributed to this metabolic switch. Based on these observations, CLL cells' acquired increased glucose dependency as well as Notch-c-Myc signaling could be therapeutically exploited in an effort to overcome stroma-mediated drug resistance. (*Blood*. 2015;125(22):3432-3436)

Introduction

B-cell chronic lymphocytic leukemia (CLL) is the most common lymphoproliferative disorder in adults. One of the hallmarks of CLL cells is their recurrent contact with the stromal microenvironment in the bone marrow and secondary lymphoid tissues.[1] This bidirectional interaction[2] has been shown to provide protection from spontaneous apoptosis.[3] Moreover, increasing evidence suggests that resistance of CLL cells toward drug-induced apoptosis is also mediated by stromal contact.[4] This stromal protection of CLL cells presumably contributes to paving the way for drug-resistant clones, minimal residual disease, and finally relapses.[5,6] Thus, it is essential to unravel the mechanisms by which the microenvironmental niches protect CLL cells in order to develop more effective therapeutic approaches.

Nowadays, a plethora of molecules including integrins,[3] spleen tyrosine kinase,[7] stromal derived factor-1,[8] Notch,[9] CD44,[10] and thioredoxin[11] have been identified to be part of the stromal cross talk, whereas bioenergetic mechanisms remain poorly understood. Recently, we found in circulating CLL cells, in contrast to many other malignant cell types, an increased mitochondrial oxidative phosphorylation, but not an increased aerobic glycolysis.[12] This metabolic phenotype was accompanied by an increased mitochondrial biogenesis that might represent a mechanistic pathway in CLL oncogenesis.[13] In the past, it has been shown that the stromal microenvironment can promote a metabolic switch in malignant cells from mitochondrial respiration away to glycolysis (eg, in acute myeloid leukemia blasts).[14] This so-called Warburg effect not only confers growth advantages but also contributes to chemoresistance.[15] We therefore sought to determine whether stromal cells have an impact on CLL cells' metabolism and which signaling pathways are involved in this process.

Study design

Patient samples

Blood samples from 49 patients (supplemental Table 1, available on the *Blood* Web site) were obtained upon approval by the Ethics Committee of the University of Erlangen-Nuremberg and upon patients' informed consent (approval number: 3779).

Cells

Peripheral blood mononuclear cells were obtained using Ficoll-Paque (GE Healthcare, Piscataway Township, NJ). $CD19^+$ cells were purified by magnetic bead-based negative selection (B-cell Isolation Kit II; Miltenyi Biotec, Bergisch Gladbach, Germany) at a purity level of ≥90%. The Notch-1 mutational analysis is detailed in the supplemental Methods. The HS-5 human bone marrow stromal cell line was purchased from American Tissue Culture Collection (Manassas, VA), and primary human lymph-node–derived fibroblasts (HLFs) were purchased from ScienCell (Carlsbad, CA). Mesenchymal stromal cells (MSCs) were isolated from iliac crest bone marrow aspirates taken from healthy donors (approval number: 200_12) and expanded as previously detailed while fulfilling uniformly the minimal MSC criteria.[16]

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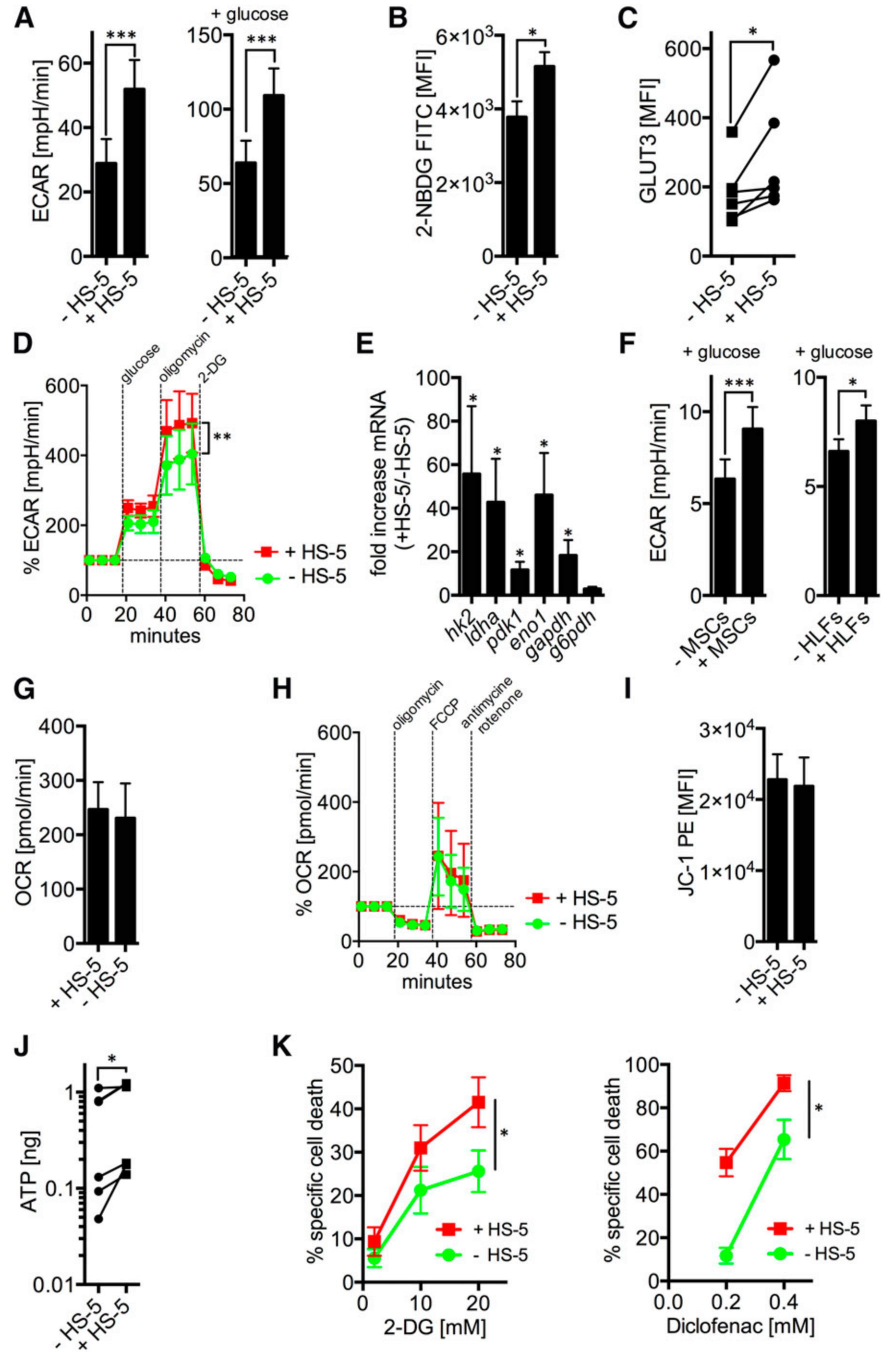

Figure 1. Bone marrow stromal cells promote aerobic glycolysis in primary CLL cells. (A) The ECAR, which quantifies proton production as a surrogate for the overall glycolytic flux was evaluated in CLL cells (n = 4) following a 6-day culture in the presence or absence of stromal contact under baseline conditions and upon glucose administration using an XFe96 flux analyzer. (B) Glucose uptake in primary CLL cells (n = 6) cultured for 6 days in the presence or absence of HS-5 as quantified by FACS based on the mean fluorescence index (MFI) of the incorporated fluorescent glucose analog 2-NBDG (2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-d-glucose). (C) Density of the glucose transporter GLUT3 was assessed on primary CLL cells (n = 6) cultured in the presence or absence of HS-5 by FACS. (D) ECAR was measured in purified primary CLL cells (n = 4) following a 6-day culture in the presence or absence of stromal contact under basal conditions, in response to glucose, and upon blocking the mitochondrial ATP generation by oligomycin. The resulting (compensatory) effects on ECAR following the interference with the mitochondrial energy metabolism represent the maximal glycolytic capacity and are shown as a percentage of the baseline measurement (set as 100%). (E) Changes in the relative gene expression of key glycolytic enzymes including hexokinase-2 (*hk2*), lactate dehydrogenase A (*ldha*), pyruvate dehydrogenase kinase-1 (*pdk1*), enolase-1 (*eno1*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), and glucose-6-phosphate dehydrogenase (*g6pdh*) were determined by qPCR in primary CLL cells (n = 5-8) upon stromal contact for 6 days as compared with cells cultured alone (set as 1). (F) ECAR was measured in response to glucose in purified primary CLL cells (n = 4-6) following culture in the presence or absence of contact to primary human bone marrow-derived MSCs from 3 healthy donors or primary HLFs from 1 donor. (G) The oxygen consumption rate (OCR) indicative for mitochondrial respiration was measured by an XFe96 flux analyzer under baseline conditions in purified primary CLL cells (n = 4) following a 6-day culture in the presence or absence of stromal contact. (H) In addition to measurements under basal conditions, OCR was assessed in response to the indicated mitochondrial inhibitors. The resulting effects on OCR are shown as a percentage of the baseline measurement (set as 100%) for each treatment. Alterations after oligomycin and FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) are indicative for respiration linked to ATP production and the maximal respiratory capacity, respectively. (I) The $\Delta\Psi M$ was semiquantified using the potentiometric dye JC-1 (Cayman Chemical) by FACS. CLL cells (n = 3) cultured with/without HS-5 cells were comparatively assessed. (J) ATP levels were measured in lysates from purified CLL cells (n = 5) cultured alone or in the presence of HS-5 cells using a colorimetric assay. (K) The % specific cell death as assessed by FACS is shown for purified CLL cells treated for 24 hours with 2-deoxy-D-glucose (2-DG) (n = 5) and diclofenac (n = 3) in the presence or absence of HS-5 cells. Bars indicate the standard error of the mean. * $P < .05$; ** $P < .005$; *** $P < .001$.

ATP levels

Adenosine triphosphate (ATP) concentration was assessed using a colorimetric ATP Assay Kit (Abcam, Cambridge, UK).

Antibodies and flow cytometry (FACS)

Cells were stained according to the manufacturer's recommendations using fluorochrome- coupled antibodies (supplemental Table 2). Cells were analyzed using a fluorescence-activated cell sorter (FACS) Canto II cytometer (BD Biosciences) and the FlowJo Version 9.5 software (TreeStar, Ashland, OR).

DNA, RNA preparation, and quantitative polymerase chain reaction (qPCR)

RNA and DNA were extracted from cell lysates (RNeasy Mini Kit; Qiagen, Hilden, Germany), and cDNA was prepared (Superscript First Strand Synthesis System; Life Technologies) using a Mastercycler Nexus (Eppendorf, Hamburg, Germany). The mRNA levels were quantified by qPCR (Quantitect SYBR Green PCR Kit; Qiagen) on a Rotor Gene Q (Qiagen). Relative gene expression was determined by normalizing the expression of each target gene to β2-microglobulin using gene-specific primers (supplemental Table 3).

Extracellular flux analysis

Bioenergetics were determined using an XF96e Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA) as previously described in detail.[12]

Statistical analyses

Differences in means were evaluated with parametric or nonparametric methods based on the distribution levels. All statistical analyses were performed using

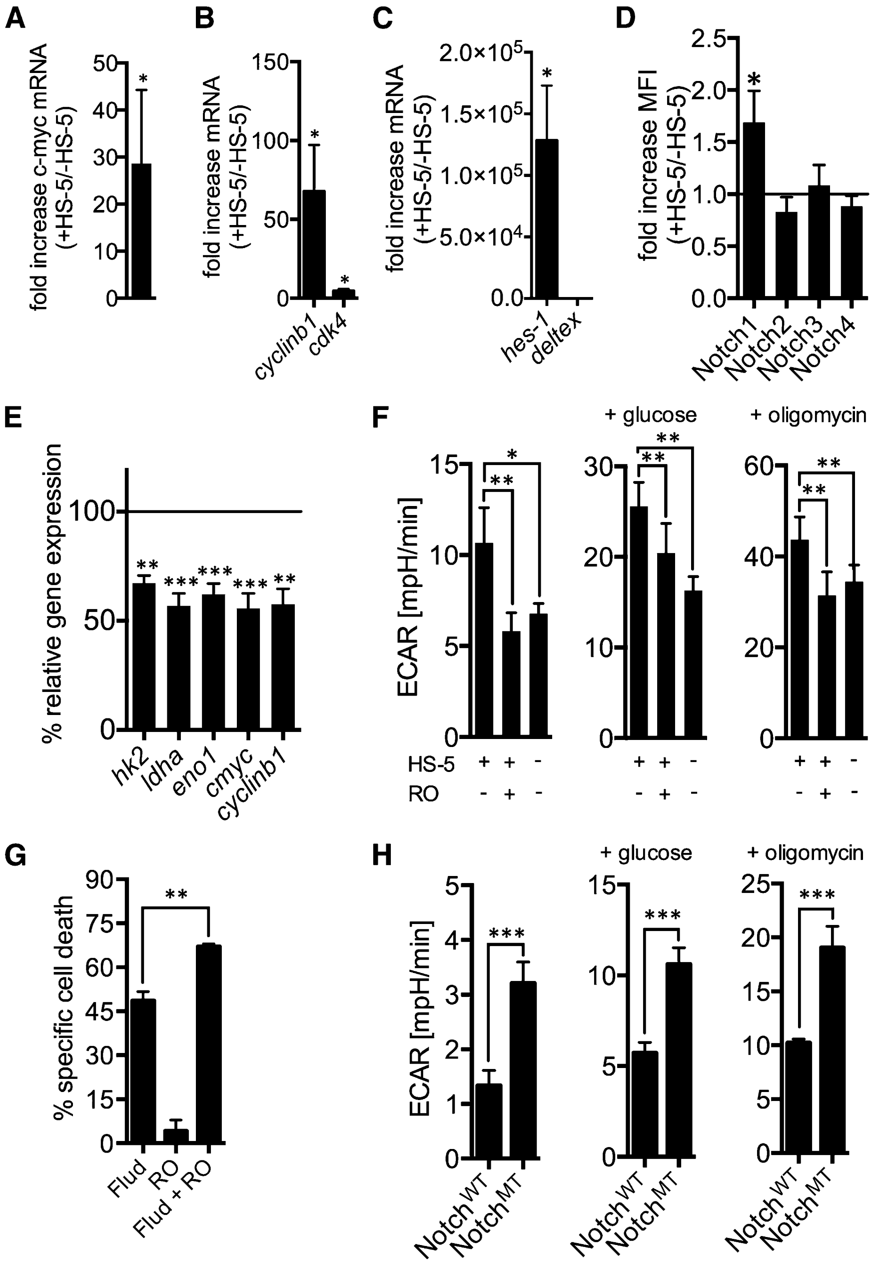

Figure 2. Notch-c-Myc signaling contributes to the stromal cell-mediated glycolytic switch. (A) Changes of the *c-myc* relative gene expression were evaluated in primary CLL cells (n = 5) cocultured with HS-5 cells for 6 days as compared with cells cultured alone (set as 1). (B) Changes of *cyclinb1* and *cdk4* relative gene expression, both c-Myc target genes, in CLL cells (n = 6) with/without stromal contact as quantified by qPCR. (C) Changes of the relative expression of genes indicative for a canonical (*hes-1*) and noncanonical (*deltex*) Notch pathway activation is shown for CLL cells (n = 6) cocultured with HS-5 cells for 6 days as compared with cells cultured alone (set as 1). (D) Upregulation of Notch receptors was evaluated by FACS in nonpermeabilized CLL cells (n = 4) cultured in the presence or absence (set as 1) of HS-5 cells. (E) Relative gene expression of glycolytic enzymes (*hk2*, *ldha*, *eno1*), of *c-myc*, and of the c-Myc target gene *cyclinb1* in primary CLL cells (n = 4-8) cocultured with HS-5 cells ± the GS inhibitor RO4929097 in a noncytotoxic dosage (2.5 μM). Expression levels in untreated cells are set as 100%. (F) The ECAR as a surrogate for glycolysis was measured in CLL cells (n = 4) using an XFe96 flux analyzer under baseline conditions, in response to glucose administration, and upon application of oligomycin, which blocks mitochondrial ATP production. CLL cells were cultured in the presence/absence of HS-5 cells and of the GS inhibitor RO4929097 (RO) as indicated. (G) Primary CLL cells (n = 4) cocultured with HS-5 stromal cells were treated with either fludarabine (Flud) or RO4929097 (RO) or both as indicated. The specific cell death was assessed by FACS. (H) The ECAR, which represents a surrogate for the overall glycolytic flux, was measured in purified primary CLL cells carrying a wild-type (WT) (n = 4) or a mutated (MT) (n = 5) Notch-1 under basal conditions, in response to glucose, and upon blocking the mitochondrial ATP generation by oligomycin. Bars indicate the standard error of the mean. * $P < .05$; ** $P < .005$; *** $P < .001$.

GraphPad Prism Version 5 (GraphPad Prism Software Inc.) at a significance level of $P < .05$.

Results and discussion

In a recent study, the human bone marrow stromal cell line HS-5 improved CLL cell survival and attenuated chemotherapy-induced cell death by regulating the cystine metabolism.[17] Applying this niche model, we confirmed an improved viability of CLL cells and a better protection against fludarabine, a chemotherapeutic widely used in CLL (supplemental Figure 1).

In order to evaluate the impact of HS-5 on glycolysis, we measured the lactic acid production via the extracellular proton release (extracellular acidification rate [ECAR]). Coculturing CLL cells with HS-5 cells led to a marked increase in ECAR under baseline conditions (51.9 ± 9.1 vs 28.82 ± 7.58 mpH/min) and upon glucose addition (109.2 ± 18.18 vs 63.87 ± 14.99 mpH/min) (Figure 1A). This glycolytic switch occurred irrespective of the immunoglobulin variable heavy-chain mutational status (supplemental Figure 2). In line with this observation, we found an increased glucose uptake accompanied by a significantly enhanced expression of the glucose transporter GLUT3 (Figure 1B-C; supplemental Figure 3).

Blocking mitochondrial ATP production by oligomycin shifts energy production toward glycolysis with an increase in ECAR that reveals the cells' maximal glycolytic capacity. In fact, stromal contact resulted in a significantly increased glycolytic capacity (425.9 ± 48.48 vs 355.1 ± 39.66%, baseline ECAR set as 100%) (Figure 1D). Consequently, expression levels of key enzymes controlling glycolysis were significantly higher in those cells including *hexokinase-2* (55.71-fold), *lactate dehydrogenase A* (42.7-fold), *pyruvate dehydrogenase kinase-1* (11.64-fold), *enolase-1* (46.08-fold), and *glyceraldehyde-3-phosphate dehydrogenase* (18.32-fold) (Figure 1E). Furthermore, coculturing CLL cells with primary bone marrow–derived MSCs or HLFs, which both hold niche functions (supplemental Figure 4), led to an increase of glycolysis (Figure 1F) accompanied by an upregulation of the key glycolytic enzyme *lactate dehydrogenase A* (supplemental Figure 5).

Baseline respiration and maximal respiratory capacity measured upon application of the uncoupling agent FCCP remained unaffected (Figure 1G-H; supplemental Figure 6). The mitochondrial membrane potential ($\Delta\Psi$M) represents a good indicator for mitochondrial functionality. Because stromal contact leads to mitochondrial depolarization in acute myeloid leukemia cells and thereby to a concomitant compensatory increase of glycolysis,[14] we measured $\Delta\Psi$M without detecting any changes (Figure 1I). Although glycolysis yields less ATP as compared with oxidative phosphorylation, the rate of production might be 100 times faster resulting in greater ATP abundance.[18] In fact, CLL cells with a glycolytic phenotype upon HS-5 coculture exhibited increased ATP levels (Figure 1J), which could contribute to chemoresistance.[19]

Despite the demonstrated tumor-promoting effects (Figure 1A), shifts of cellular energetics away from oxidative phosphorylation could increase the CLL cells' reliance on glucose and thereby render them more susceptible toward perturbations within their glycolytic framework.[20] In fact, treating CLL cells with 2-deoxy-D-glucose and diclofenac[21] that both target glucose metabolism revealed an increased sensitivity upon stromal contact (Figure 1K).

The c-Myc protooncogene holds a central role in regulating tumor growth and metabolism.[22] CLL cells cocultured with HS-5 (or with MSCs/HLFs) displayed a significant upregulation of c-Myc gene and protein expression (Figure 2A; supplemental Figures 7 and 8), which is in line with recent in situ observations suggesting a microenvironmental c-Myc activation in CLL.[23] The efficient triggering of the c-Myc pathway is highlighted by the significant increase of the according target genes[24] *cyclinb1* (67.66-fold) and *cdk4* (4.54-fold) (Figure 2B; supplemental Figure 9). Testing various (co-)culture settings revealed that the observed alterations in gene expression were cell contact dependent (supplemental Figure 10).

Recent evidence implicates the contribution of Notch signaling to the stromal cell–mediated effects.[9] HS-5 cells express the ligands Jagged-1/2 that bind Notch-1 to Notch-4 receptors found on CLL cells, and delta-like-3 is additionally found on primary MSCs and HFLs (supplemental Figure 11). In fact, stromal contact led to an upregulation of *hes-1* (not *deltex*) expression reflecting a canonical Notch activation and of the Notch-1 receptor in CLL cells (Figure 2C-D; supplemental Figure 12). Notably, Notch signaling can directly activate c-Myc.[25] Ligand binding leads to a γ-secretase (GS)–mediated cleavage and subsequent nuclear translocation of the Notch intracellular domain. Treating CLL cells with a GS inhibitor in noncytotoxic but still effective dosages (supplemental Figure 13) significantly diminished the stromal-mediated upregulation of *c-myc* and its target gene *cyclinb1* as well as of the tested glycolytic enzymes (Figure 2E). Functionally testing the effects of the GS inhibition revealed an abolishment of the glycolytic shift (Figure 2F) further corroborating the involvement of the Notch-c-Myc axis. Inhibiting GS had a synergistic effect on the fludarabine-induced cell death on CLL cells under stromal contact (Figure 2G). CLL cells harboring a mutated Notch-1 exhibit an increased Notch pathway activation as compared with their wild-type counterparts.[26] In line with our observations, Notch-1–mutated CLL cells showed increased glycolytic parameters (Figure 2H; supplemental Figure 14).

Taken together, our findings suggest a microenvironmental glycolytic shift in CLL cells mediated by Notch-c-Myc signaling. Interfering with this pathway or the glucose metabolism in CLL cells could be therapeutically exploited for targeting the stromal niche's protective effects and remains to be further elucidated.

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Authorship

Contribution: R.J. performed research, analyzed data, and helped to write the manuscript; M.B., M.Q., and D.S. performed research; K.L.B. and T.Z. provided material and performed research; and D.M. designed the study, analyzed data, and wrote the manuscript.

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