Original Article

Tumor Biology

Tumor Biology
February 2017: 1–9
© The Author(s) 2017
Reprints and permissions:
sagepub.co.uk/journalsPermissions.nav
DOI: 10.1177/1010428317694325
journals.sagepub.com/home/tub
SAGE

Plasma matrix metalloprotease 9 correlates with blood lymphocytosis, leukemic cell invasiveness, and prognosis in B-cell chronic lymphocytic leukemia

Milena Gusella[1], Caterina Bolzonella[1], Rossella Paolini[2], Elisabetta Rodella[2], Laura Bertolaso[1], Cinzia Scipioni[3], Silvia Bellini[3], Antonio Cuneo[4], Felice Pasini[1] and Emilio Ramazzina[2]

Abstract

The complex biology underlying chronic lymphocytic leukemia cell migration and tissue invasiveness is not yet completely understood and might provide novel predictive markers and therapeutic targets. A total of 36 patients out of treatment from at least 3 months were enrolled and followed up for a median period of 44.2 months (range: 4.4–99.2). Matrix metalloprotease 9 and tissue inhibitor of metalloproteases 1 plasma levels and production/release from lymphoid cells were measured by zymography and enzyme-linked immunosorbent assay (ELISA) analysis. Malignant and normal lymphocyte mobility and matrix-degradation capability were studied using a Boyden chamber system, with and without autologous plasma. Free matrix metalloprotease 9 plasma levels were related with blood lymphocytosis, especially in more advanced stages ($p = 0.003$), and higher concentrations were associated with an increased disease progression risk (hazard ratio = 9.0, 95% confidence interval = 1.5–13.8). Leukemic cells expressed and secreted very little matrix metalloprotease 9. On the contrary, normal lymphocytes derived from the same leukemic patients showed matrix metalloprotease 9 intracellular levels that were lower in subjects with higher blood lymphocytosis ($p = 0.024$) and more advanced stages ($p = 0.03$); the released quantities were inversely associated with matrix metalloprotease 9 plasma concentrations ($p = 0.035$). Leukemic cells had a reduced spontaneous mobility and matrix-degradation capability that were stimulated by autologous plasma ($p = 0.001$) and normal lymphocytes ($p = 0.005$), respectively. Matrix metalloprotease 9 affected cell invasiveness depending on concentration and disease stage. In conclusion, chronic lymphocytic leukemia cells have a reduced mobility, matrix-degradation capability, and matrix metalloprotease 9 production compared to their own autologous normal lymphocytes. They are exposed to matrix metalloprotease 9 of prevalently systemic origin whose higher levels are associated with both leukemic and normal lymphocyte accumulation in the peripheral blood and have a negative prognostic value.

Keywords

Chronic lymphocitic leukemia, matrix metalloproteinases, cell trafficking, prognosis

Date received: 10 October 2016; accepted: 23 December 2016

[1]Department of Oncology, Azienda ULSS 18 Rovigo, Rovigo, Italy
[2]Department of Medicine, Azienda ULSS 18 Rovigo, Rovigo, Italy
[3]Department of Transfusion Medicine, Azienda ULSS 18 Rovigo, Rovigo, Italy
[4]Department of Medical Sciences, Section of Hematology, University of Ferrara, Ferrara, Italy

Corresponding author:
Milena Gusella, Department of Oncology, Azienda ULSS 18 Rovigo, Santa Maria della Misericordia Hospital, Viale Tre Martiri, 140, 45100 Rovigo, Italy.
Email: milenagusella@libero.it

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by neoplastic lymphocyte (Ly) accumulation in the peripheral blood and infiltration of lymphoid tissues and bone marrow. Leukemic cell trafficking has a pivotal role in disease pathogenesis and progression. The constitutive CLL cell mobility is known to be reduced compared to normal Ly[1,2] and affected by a matrix metalloprotease (MMP), the isoform pro-MMP9.[3,4] Higher MMP9 levels were reported in patients' serum,[5] inside leukemic cells,[6] and in their culture medium.[5] According to its matrix-degradation (MD) activity, MMP9 seemed associated with tissue invasiveness.[6] Exogenous or endogenous MMP9 could bind to CLL cell surface through an alpha4-beta1 integrin/CD44v complex, that is absent in normal B cells.[7] In vitro, cell surface–bound MMP9 increased CLL cell trans-migration that was reduced by specific MMP9 inhibitors[6] or MMP9 gene silencing.[3] But surprisingly, it was also found that pre-incubation with high concentrations of pro-MMP9 (>27 nM) inhibited primary CLL cell transmigration.[7] The phenomenon was confirmed in vivo, by injecting CLL viable cells pre-incubated with pro-MMP9 into immunedeficient mice and finding a reduced cell homing into lymphoid tissues starting from 30 nM of pro-MMP9 onward.[8] In addition, binding of high levels (>27.5 nM) of pro-MMP9 to CLL cell surface significantly counteracted spontaneous apoptosis in vitro; accordingly, CLL cells infiltrating bone marrow and lymph nodes had an increased drug-resistance and expressed more surface MMP9 than their counterparts from peripheral blood.[9] Finally, MMP9 serum levels were correlated to disease prognosis.[10,11]

All these data suggested that MMP9 could influence CLL cell trafficking and prognosis. But a major problem was that in vitro levels of MMP9 necessary to influence CLL cell behavior were considerably higher[7,9] compared to those found in patients.

In addition, a MMP9-specific inhibitor, the tissue inhibitor of metalloproteases 1 (TIMP1), is physiologically secreted: its role in CLL cell migration, MD, and disease progression is not completely known. Moreover, numerous extrinsic factors affected CLL cell trafficking, such as different chemokines and a variety of corresponding receptors or integrines with interindividual variable expression on the CLL cell surface,[12] but their real clinical effect in CLL cell blood accumulation and extravasation is still unclear, as well.

MMPs are considered promising in the area of targeted therapies: they are associated with angiogenesis, invasion, and metastasis in different types of solid cancer, and MMP inhibitors have been developed for the cancer treatment.[13] Paucity of data is available in the field of B-cell chronic lymphocytic leukemia (B-CLL), and a more in-depth knowledge is necessary to define MMP9 pathological roles and whether its inhibition may be beneficial or has to be avoided.[14]

The aim of the study was to evaluate whether MMP9/TIMP1 is associated with CLL lymphoid cell mobility, invasion capability, and disease prognosis. Our experimental model tried to reproduce the physiological condition as far as possible, with reference to the relationship between lymphoid subpopulations and exposure to soluble factors contained in extracellular fluids.

Methods

The study was approved by the local ethics committees; all patients gave their signed informed consent.

Lymphocyte isolation

Peripheral blood samples were collected in heparined tubes from patients fulfilling clinical and immunophenotypic criteria for CLL; they should be out of specific treatment from at least 3 months. As a control, blood samples from healthy subjects were also used.

Lymphocytes (Ly) were isolated via density gradient centrifugation (Biocoll Separating Solution 1.077 g/mL; Biochrom, Berlin, Germany) and negatively enriched by plastic adhesion. The resulting samples consisted of CLL cells (media concentration 70.9%, range: 51.3%–92.6%) together with normal T (18.7%, 4%–32.7%) and B (10.4%, 3.4%–17.9%) cells, resembling the individual ratios in peripheral blood; they were referred to as Whole-Ly (WL) samples. A part of each WL sample was further depleted of normal Ly, using B-Cell Isolation kit in a MidiMACS Separator (Miltenyi Biotec, Auburn, CA, USA), and obtained highly purified samples consisting of 95.4% (range: 93%–97.7%) of leukemic cells, referred to as CLL–Ly (CL) samples. Flow-cytometry was performed to characterize sample compositions; antibodies against CD45, CD19, CD5, and CD3 (Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA) were used soon after isolation from blood and after 48 h of culture.

MMPs and TIMP1 detection

MMP9 gene expression was determined on total RNA, extracted from Ly following the phenol-chloroform protocol and reverse-transcripted with Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) and random primers (Invitrogen, ThermoFisher Scientific, Whaltham, MA, USA). Quantitative polymerase chain reaction (PCR) was conducted on a 7500 real time PCR system (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) using power SYBR green master mix (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA). Relative MMP9 gene expression was calculated by $\Delta\Delta$Ct method, using 36B4 and SNRPD3 as housekeeping genes.

MMP9 protein intracellular content was determined immediately after blood withdrawal; briefly, 5×10^6 cells were lysed on ice with 2%Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 5 mM ethylenediaminetetraacetic acid (EDTA), 7 KIU/mL Aprotinin, 1 μg/mL Pepstatin, 1 μg/mL Leupetin, and 10 mM phenylmethane sulfonyl fluoride (PMSF) in PBS. MMP9 content was expressed as pg/100 μg protein. From another aliquot of the same sample, 5×10^6 cells were seeded in 1 mL of RPMI medium plus 0.1% Albumine (Al) and incubated at 37°C and 5% CO_2; MMP9 secretion capacity was estimated by measuring MMP9 concentration in the conditioned medium after 48 h of in vitro culture.

MMP9 in plasma, cell lysates, and 48 h-conditioned medium was analyzed and quantified by zymography. Briefly, 20 μL of each sample was loaded into a 7.5% SDS–polyacrilamide gel containing 0.1% of type A gelatin. After electrophoresis, the gel was washed three times with 2.5% Triton X-100 for 30 min and then incubated overnight at 37°C with a development buffer (Zymogram Development Buffer, Bio-Rad), which provided optimal conditions for the MMPs activity. The day after, the gel was stained with a solution of 0.5% Coomassie brilliant blue, 30% methyl alcohol, and 10% acetic acid for 3 h and destained with 30% methyl alcohol and 10% acetic acid for three washes, for 15, 30, and 60 min. The gelatinase activity of MMP9 was detected as clear bands on a blue background. For the quantitative zymography, the optical density of the MMP9 sample bands was measured with NIH 1.62 Image program and compared with the optical density of the MMP9 purified standard bands, loaded in the same SDS-PAGE at known scalar concentrations. TIMP1 concentration was measured in plasma samples and 48-h conditioned medium with the ELISA kit Quantikine Human TIMP1 Immunoassay (R&D Systems), according to manufacturers' instructions.

Cell mobility and invasiveness

Ly mobility and invasion capability were assayed on freshly isolated cells. A two chamber trans-well system was applied, according to a Boyden strategy. Cell mobility was defined as the percentage of cells able to pass through trans-well plastic inserts with 8 μm-holes (BD Falcon Cell Culture Inserts). Cell invasiveness was estimated with the percentage of cells which migrated through a layer of a protein mix, called Matrigel, resembling basal membranes and extracellular matrix, coated on trans-well porous inserts (BD BioCoat Growth Factor Reduced MATRIGEL® Invasion Chambers). Briefly, 5×10^5 Ly were seeded in the upper compartments and the systems were incubated for 48 h at 37°C in 5% CO_2. Cells which migrated in the lower compartments were counted and characterized with flow-cytometry.

MD capability was estimated through an index (matrix-degradation index (MDI)) obtained as a ratio between the number of cells which migrated through Matrigel and the number of cells of the same sample which migrated through insert alone. Two different experimental conditions were used: (1) plain medium (with no specific chemo-attractant factor) consisting of RPMI plus 0.1% Al and (2) plasma-enriched medium, consisting of RPMI plus 5% or 10% of patients' plasma.

Statistical analysis

Correlations were analyzed with linear regression or Spearman analysis for continuous and categorical variables, respectively. Differences between groups were evaluated with Students' t test or analysis of variance (ANOVA). Welch correction was used in the case of different variances and Kruskal–Wallis option in the case of non-Gaussian distribution.

The impact of MMP-/TIMP1 on disease clinical outcome was evaluated using disease progression as an endpoint: progression free survival (PFS) was defined as the time from study entry until objective worsening of clinical conditions, demonstrated by upstaging and/or start of a specific treatment. Log-rank test was used to compare survivals; hazard ratio (HR) and 95% of its confidence interval (CI) were also reported.

Results

A total of 36 patients (20 males) affected by B-CLL were enrolled, median age was 66.0 years (range: 36–85). Three had been previously treated; at the time of study entry, all patients were out of treatment from at least 3 months.

Median peripheral Ly count was $14 \times 10^3/\mu L$ (range: 5.2–81.7); a median of $8.2 \times 10^3/\mu L$ (4.4–75.7) were leukemic while $4.5 \times 10^3/\mu L$ (2.5–6.3) were normal Ly. A significant positive correlation between the two lymphoid subpopulations was found: according to median value, higher CLL cell counts were accompanied by significantly higher normal Ly counts (3.4×10^3 vs $5.2 \times 10^3/\mu L$, $p = 0.003$, in Supplementary Material Figure S1).

Patients were clinically staged according to the RAI system as stage 0 (n = 12), stage 1 (15), stage 2 (6), and stages 3 and 4 (3). After a median follow-up of 44.2 months (range: 4.4–99.2), 15 patients (42%) showed an objective worsening of the disease and received a specific treatment. Seven patients died of the disease. The RAI classification was associated with PFS from study entry ($p < 0.001$) and overall survival ($p = 0.002$; data not shown).

MMP9 and TIMP1 plasma levels and CLL prognosis

Plasma samples showed homogeneously high levels of pro-MMP2, both in patients and healthy subjects; on the contrary, pro-MMP9 had marked levels in CLL patients compared to healthy subjects, with a median concentration

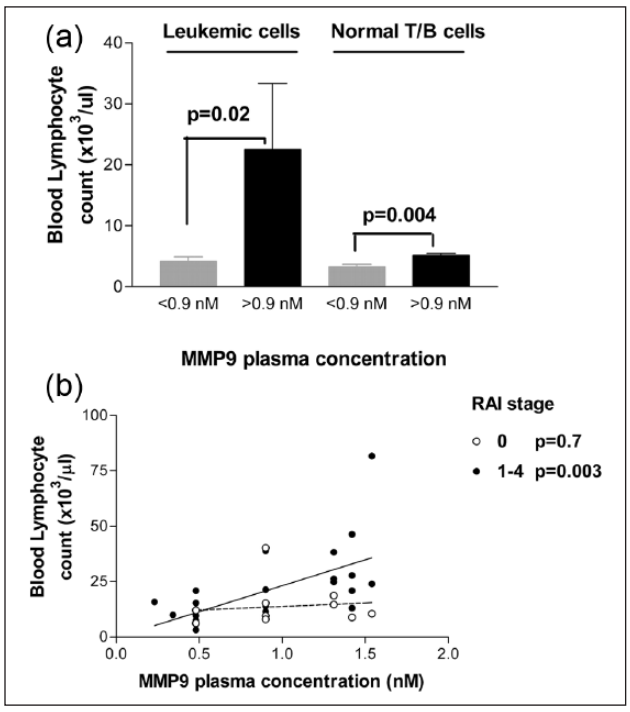

Figure 1. Association of pro-MMP9 plasma levels with lymphocyte counts, for (a) both CLL cells and normal lymphocytes or (b) in different RAI stages (0, broken lines, 1–4, black line).

of 0.9 nM (range: 0.23–1.53) and an interindividual variation of 6.7 folds (in Supplementary Material Figure S2).

TIMP1 plasma levels had a mean value of 3.98 ± 1.55 nM (interindividual variation of 4.2 folds). There was no statistically significant relationship between pro-MMP9 and TIMP1 plasma levels (p = 0.7). However, in each patient, TIMP1 concentration exceeded pro-MMP9 concentration (the molar ratio ranged from 2 to 12).

Elevated pro-MMP9 concentrations (⩾median value) were associated with higher leukemic counts (22.5 ± 20.5 vs 4.2 ± 1.4 × 10^3, p = 0.02) and normal Ly counts (5.2 ± 0.8 vs 3.3 ± 0.8 × 10^3, p = 0.004; Figure 1(a)). Moreover, a significant linear correlation was found between total circulating lymphoid cells and pro-MMP9 plasma levels (Pearson r = 0.48, p = 0.004). Interestingly, this latter relationship changed according to RAI stage: it was not significant in stage 0 (r = 0.13, p = 0.7) and highly significant in stages 1–4 (r = 0.60, p = 0.003; Figure 1(b)).

Finally, a correlation was found between pro-MMP9 plasma levels and PFS: patients with concentrations ⩾pro-MMP9 median value showed a significantly increased risk of disease progression compared to patients with low levels (HR = 9.0, 95% CI = 1.5–13.8; median PFS 34.3 months vs not reached). After 36 months of follow-up, only one out of eight patients (12.5%) with low pro-MMP9 experienced disease progression compared with 14 out of 18 (77.8%) in the patient subgroup with high pro-MMP9 (p = 0.03, Figure 2).

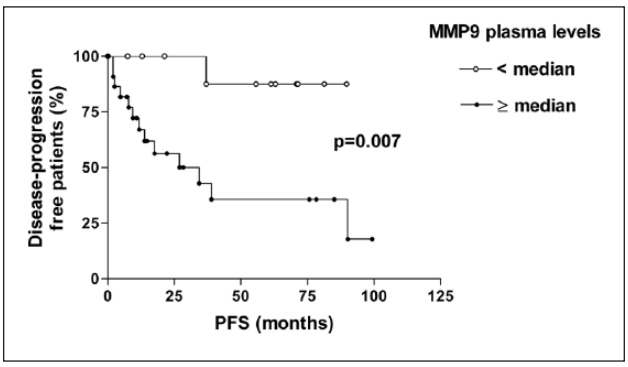

Figure 2. Association between pro-MMP9 plasma levels and progression free survival.

No relationships between plasma pro-MMP9 or TIMP1 levels and RAI classification were found. No association between PFS and TIMP1 plasma levels (p = 0.17) or MMP9/TIMP1 ratio (p = 0.12) was detected.

MMP9 and TIMP1 lymphocyte production and secretion

In order to evaluate whether pro-MMP9 free in the blood might derive from circulating lymphoid cells, their capability of spontaneous release was analyzed in vitro. WLs secreted relatively high quantities of pro-MMP9 (mean release: 7.9 ng/10^6 cells, range: 0.3–40) that were inversely associated with leukemic cell percentage in the sample (p = 0.007, Figure 3(a)). Accordingly, CLs tested alone did not secrete detectable levels of pro-MMP9.

Intracellular pro-MMP9 was also measured: freshly isolated WLs contained pro-MMP9 ranging from 0.24 to 45.6 ng/100 ng of protein (190-fold variation). Again, a negative correlation was found between pro-MMP9 cell content and leukemic cell percentage in the sample (p = 0.009, Figure 3(b)) suggesting that pro-MMP9 was produced principally by healthy Ly. Intracellular pro-MMP9 levels of normal Ly were associated with blood lymphocytosis: mean cell content was 5.2 ± 5.5 and 1.6 ± 0.9 ng/100 ng protein/10^6 cells in patients with blood Ly counts less and more than 20 × 10^3/μL, respectively (p = 0.024). Moreover, intracellular pro-MMP9 in normal Ly decreased in relation to RAI stage: median value was 5.6 ng/100 ng protein/10^6 cell (range: 2.0–22.3) for patients in stage 0, 3.4 (0.8–11.2) in stage 1, and 1.3 (0.2–2.8) in stages 2–4 (p = 0.03).

As a confirmation for pro-MMP9 production, MMP9 messenger RNA (mRNA) was measured in fresh samples and resulted a hundred folds less expressed in CLs than in WLs (p = 0.002, Supplementary Material Figure S3). MMP9 mRNA levels significantly correlated with MMP9 protein concentration in 48 h-conditioned medium (p = 0.008; data not shown).

To evaluate if pro-MMP9 produced by circulating Ly could significantly contribute to plasma pro-MMP9, the

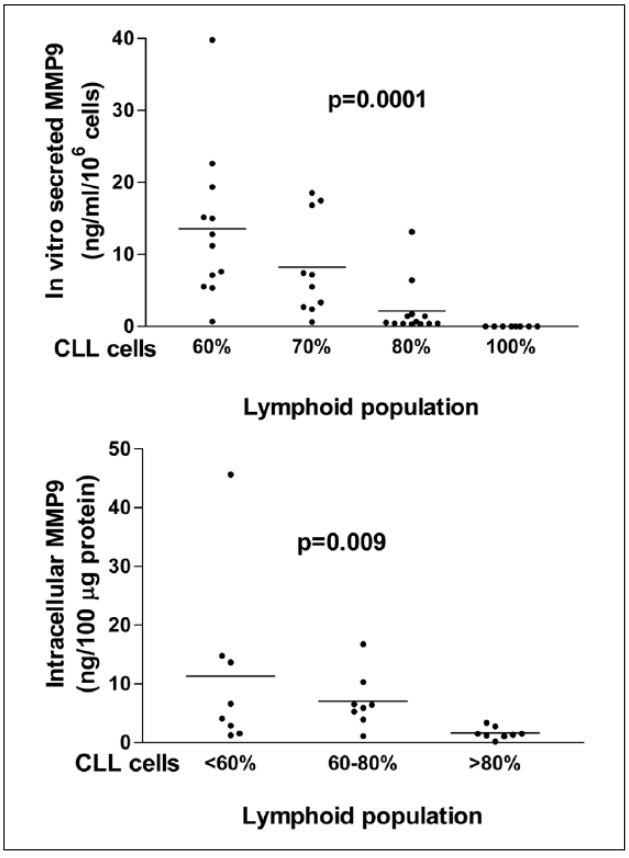

Figure 3. Relationship between percentage of leukemic cells and pro-MMP9 (a) in vitro secretion or (b) fresh sample contents.

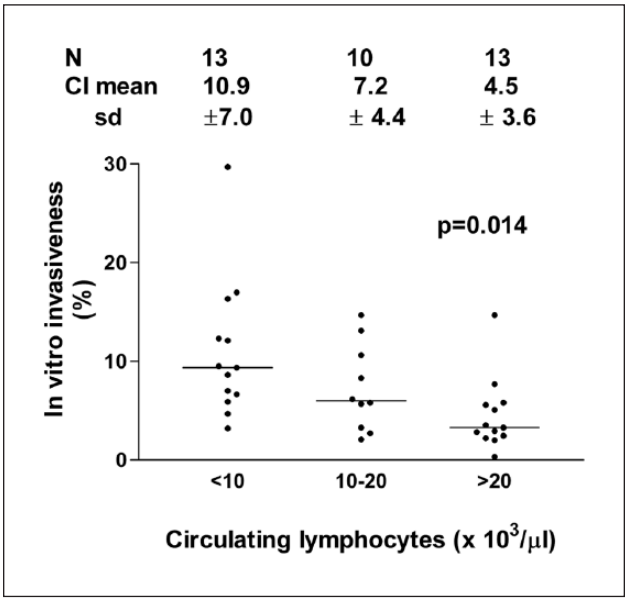

Figure 4. Inverse association between whole-lymphocyte sample in vitro invasive capability and patients' lymphocytosis. CI: cell invasiveness%.

correlation was analyzed between in vivo levels and in vitro secretion, and an inverse association emerged ($r = -0.38$, $p = 0.035$). Finally, low concentrations of TIMP1 (mean ± standard deviation = 0.16 ± 0.26 nM) were found in conditioned medium from WLs, with 15 cases (55%) that did not secret any TIMP1 at all.

Lymphocyte constitutive invasiveness and motility

Constitutive invasion capacity (IC) was estimated through the percentage of cells which migrated through a Matrigel stratum in a plain medium: in WL samples, a median value of 7.4% (range: 0.3%–29.7%) and a significant inverse correlation with patients' blood lymphocytosis ($p = 0.014$, Figure 4) were shown.

Invasiveness was also measured in the different lymphoid subpopulations (Table 1): leukemic cells alone had a significantly lower IC compared to normal autologous Ly (mean 2.8 ± 2.3% vs 18.0 ± 14.9%, paired t test $p = 0.002$). When leukemia cells were tested in co-culture with autologous normal Ly, their IC slightly but significantly increased to 4.4 ± 1.5% ($p = 0.04$, in Supplementary Material Figure S4).

In order to better characterize cell migration, two factors that independently contribute to cell invasiveness were further analyzed: cell motility (CM) and MD capability. WL median CM was 22.2% with a marked interindividual variability (range: 6.3%–55.9%). Also, it was associated to RAI stages: lymphoid CM in stages 2–4 was significantly reduced compared to stages 0–1 (17.3 ± 8.8 vs 24.1 ± 8.9, $p = 0.04$). Leukemic cells tested alone and in co-culture with autologous normal Ly had a similar mean CM of 18.5 ± 4.3% and 16.1 ± 5.1%, respectively; normal homologous Ly had a significantly higher mobility, with a mean value of 44.0 ± 10.0% (paired t test $p = 0.0003$, in Supplementary Material Figure S5).

Regarding MD capability, the MDI median value for WLs was 0.37 with a wide range of variation (0.03–1.0); compared with normal homologous Ly, leukemic cells showed the lowest MDI when tested alone (mean 0.15 ± 0.09 vs 0.47 ± 0.33, $p = 0.01$), but their MDI doubled in co-culture (0.30 ± 0.15, $p = 0.005$, in Supplementary Material Figure S6).

In order to verify whether MMP9 production may directly influence lymphocyte migration, relationships between pro-MMP9 levels in culture medium and indices of mobility or invasiveness were analyzed. A significant association was found only when a cut-off at 33rd percentile of pro-MMP9 concentrations (0.01 nM) was used: WL samples with low pro-MMP9 secretion moved less through inserts (17.6 ± 6.4 vs 24.4 ± 9.7%, $p = 0.025$) or Matrigel (4.8 ± 3.4 vs 9.2 ± 6.4%, $p = 0.03$) than those with high secretion. On the contrary, there was no association of pro-MMP9 levels, mobility, invasiveness, and degradation capability with patients' gender or age.

Table 1. Summary of means (range) for invasiveness, mobility, and matrix degradation capability assayed in vitro with a Boyden chamber system.

	Invasiveness, %	p	Mobility, %	p	Matrix degradation index	p
Leukemic cells alone	2.8 (1.0–7.6)	0.04*	18.5 (11.0–23.8)	ns*	0.15 (0.05–0.32)	0.005*
Co-cultured						
Leukemic cells	4.4 (1.6–6.1)	0.002°	16.1 (8.8–21.9)	0.003°	0.30 (0.07–0.48)	0.005°
Normal lymphocytes	18 (7.5–34.3)		44.0 (29.7–54.6)		0.47 (0.22–1.0)	
Whole lymphoid cells						
Without plasma	7.4 (0.3–29.7)	ns	22.2 (6.3–55.9)	0.001§	0.37 (0.03–1.0)	0.0008§
+5% plasma	7.5 (0.9–37.8)	§	51.9 (29.9–84.6)		0.14 (0.02–0.51)	
+10% plasma	8.5 (1.0–27.3)		58.0 (27.7–79.4)		0.12 (0.02–0.36)	

Leukemic cells from peripheral blood of CLL patients were analyzed alone or together with their own normal autologous lymphocytes, counted separately or as a whole, with or without autologous plasma.
*p in comparison between leukemic cells alone and co-cultured.
°p in comparison between leukemic cells and normal lymphocytes in co-culture.
§p in comparison among whole lymphoid samples cultured with or without plasma.

Autologous plasma effects on CLL lymphoid cell migration, invasiveness and lymphocytosis

B-CLL cell trafficking is known to be influenced by various chemokines and cytokines, with different and sometimes opposing effects; to evaluate whether blood soluble factors may influence leukemic cell mobility and invasiveness, by retaining them in vessels or stimulating extravasation, plasma was obtained from each patient and in vitro added to autologous cells. Compared with constitutive capabilities observed in plain medium, autologous plasma induced a marked dose-dependent increase of motility (Table 1): WL migrated through porous inserts were 22% without plasma and 52% ($p < 0.001$) or 58% ($p < 0.001$) with the addition of 5% or with 10% of plasma, respectively (Figure 5(a)). Pro-MMP9 (Figure 5(b)) and TIMP1 concentrations did not influence this increase.

On the contrary, invasiveness globally did not seem to change from plain condition by adding 5% or 10% of autologous plasma (mean 7.4% vs 7.5% vs 8.5%, respectively, $p = 0.8$); consequently, calculated MD capability resulted highly impaired: MDI decreased from 0.37 (range: 0.03–1.0) without plasma to 0.14 (0.02–0.51, $p = 0.001$) and 0.12 (0.02–0.36, $p = 0.0008$) when 5% or 10% plasma was added (Figure 6). However, when splitting samples according to median pro-MMP9 plasma concentration (0.1 nM), two opposite phenomenon did appear: plasma-enriched medium with low pro-MMP9 increased WL invasiveness of 60% while medium with higher pro-MMP9 levels inhibited cell migration through the Matrigel of 20% ($p = 0.03$, Figure 7(a)). The relationship between cell invasiveness and MMP9 levels was further analyzed according to RAI classification: it emerged that a strong inhibitory effect could be seen in more advanced stages (0–1 vs 2–4, $p = 0.008$; Figure 7(b) and (c)).

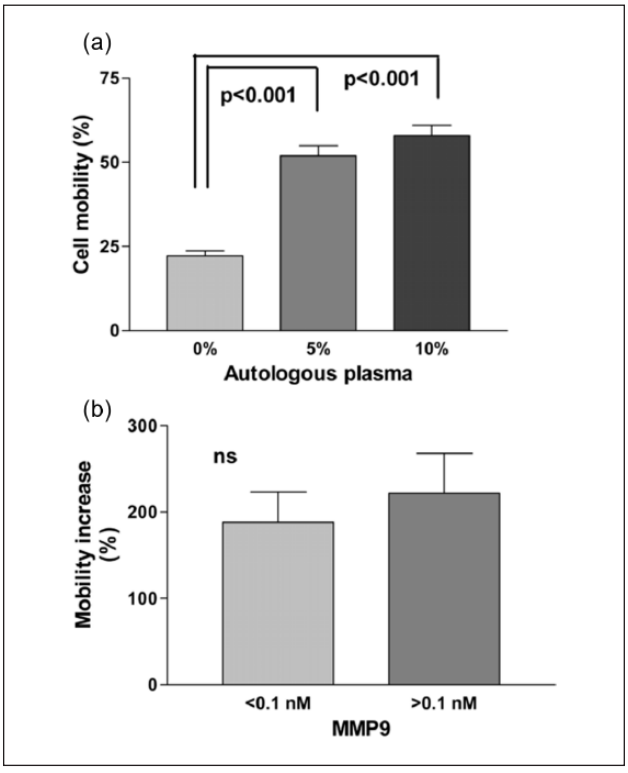

Figure 5. In vitro cell mobility with different concentrations of (a) autologous plasma and (b) pro-MMP9.

Discussion

The aim of our study is to investigate pro-MMP9/TIMP1 production in CLL and its effects on lymphoid cell invasiveness and patients' clinical features. We found that pro-MMP9 plasma levels were highly variable and related to prognosis: patients with concentrations higher than 0.9 nM (the median value of our series) had a 6-fold higher risk of disease progression (Figure 1(c)). A previous paper

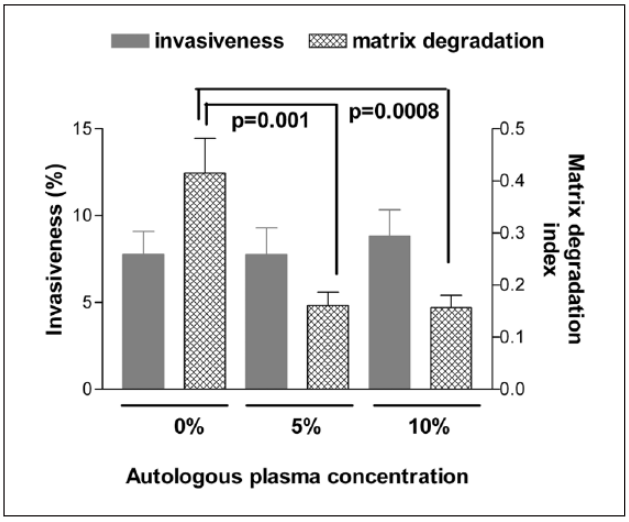

Figure 6. In vitro CLL patients' lymphoid cell invasiveness and matrix-degradation capability with different concentrations of autologous plasma.

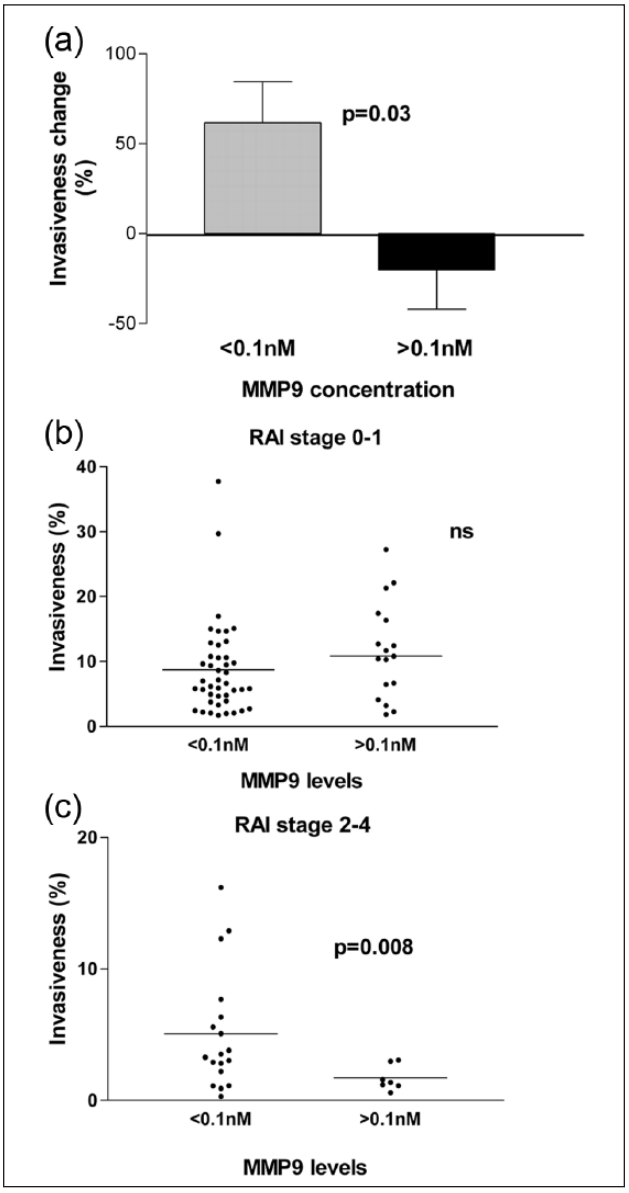

Figure 7. CLL patients' lymphoid cell in vitro invasiveness according to MMP9 concentrations (a) in all samples, or (b) in RAI stages 0–1 and (c) stages 2–4.

reported a significant correlation between MMP9 levels and PFS; however, MMP9 concentrations had been measured in serum.[10] Serum metalloproteases are prevalently derived from platelets during clotting phases, are likely different from those freely present in plasma, which are able to directly influence circulating cell migration. Consistently, our MMP9 plasma concentrations were lower than those reported in serum (mean 0.85 vs 3.2 nM), but the correlation with disease progression risk was maintained.

In our series, there was a significant linear association between plasma MMP9 concentrations and blood Ly counts, mainly due to a strong correlation in advanced stages (Figure 1(b)) that are characterized by clinically evident lymphoid tissue invasion, compared to RAI stage 0. A concordant relationship was found analysing in vitro invasiveness: In stages 0–1, lymphoid cells migrated through a matrix layer independently from medium MMP9 concentration, while in stages 2–4, there was a significantly reduced migration in presence of MMP9 higher levels (Figure 7(b) and (c)). To our knowledge, this is the first evidence of a different sensitivity of CLL lymphoid cells to MMP9 in the different stages; also, this finding underlines the difficulties of interfering with such a variably tuned mechanism from a therapeutic point of view.[15]

Differently from previous works,[5,6] we found that primary CLL cells constitutively secreted little pro-MMP9 (Figure 3(a)) and expressed low levels of MMP9 mRNA (Supplementary Material Figure S3) and protein (Figure 3(b)). However, MMP9 could be synthesized and spontaneously released by normal Ly isolated from peripheral blood of the same CLL patients. Of note, a significant inverse association existed between Ly-secreted and plasma MMP9, suggesting that circulating MMP9 was not directly derived from lymphoid cell but probably had different sources. It could derive from the tumoral microenvironment or from a systemic general inflammatory reaction to the disease;[14] in this way, circulating MMP9 levels was indicative of disease activity, and this could explain their direct association with the progression risk.

Interestingly, healthy Ly MMP9 production decreased with higher lymphocytosis and in more advanced stages, suggesting a negative effect of leukemic load on the normal Ly compartment. The observation is consistent with the already reported functional deficiencies of T cells in CLL, including reduced cytokine production, decreased

proliferation,[16,17] and impaired immunologic reactivity,[18] that worsen with disease progression.[19]

Regarding the endogenous inhibition of MMP9, we found that circulating TIMP1 levels were similar to healthy subjects and had no association with lymphoid cell blood accumulation, disease stage, and prognosis. While all lymphoid cell samples produced and secreted MMP9, the secretion of the inhibitor TIMP1 was demonstrated in around 45% of cases and was always low, supporting a marginal role in B-CLL physiopathology.

Trafficking and migration are pivotal in Ly physiology; we observed in vitro that CLL cells only partially retained these capabilities, with a high variability (10-fold interindividual variation); our data suggested that progressive accumulation in the blood was accompanied by an increasing loss of mobility and invasiveness, together with the reduction in MMP9 production and excretion. These findings agreed with Kamiguti et al.,[6] who demonstrated that CLL bone marrow was characterized by leukemic cells clearly positive for MMP9 in the first phases of the diffuse involvement, but when bone marrow became almost completely replaced by CLL cells, little MMP9 was still detectable. Again in our work, spontaneous cellular MMP9 synthesis and release were not proportionally linked to mobility and invasiveness, suggesting that these different activities, although complementary, may have different regulatory pathways. We found that only when pro-MMP9 secretion was highly impaired (under the 33rd percentile, <0.01 nM), it was also combined with a significantly lower spontaneous migration.

We also observed that leukemic cell invasiveness/MD could be significantly increased by the presence of autologous normal Ly (Table 1, Supplementary Material Figures S4 and S6). Also, soluble endogenous molecules in plasma interfered with cell mobility (Figure 5(a)) and invasiveness (Figure 6). To our knowledge, this is the first demonstration that autologous plasma is involved in leukemic cell trafficking: in vitro, addition of 5%–10% of plasma stimulated mobility (probably due to chemo-active molecules) and reduced MD capability (independently from TIMP1 presence). The considerable plasma effect in promoting Ly mobility was not related to MMP9 concentrations. On the contrary, 10%-diluted autologous plasma containing MMP9 <0.1 nM (the median value) stimulated invasiveness while that with higher MMP9 levels reduced invasiveness. This double effect was consistent with previous reports.[7] However, we argue that free pro-MMP9 that is contained in plasma prevalently exerts an inhibitory effect on lymphoid cell extravasation, because its concentrations in vivo range from 0.2 to 1.5 nM and are superior to the levels (<0.1 nM) that in vitro produced a stimulatory action.

Globally, some inconsistencies emerged between our work and previous reports[5,6] that could be due to different study settings. We used an experimental model preserving the individual leukemic/normal cell subpopulation ratios and the complexity of micro-environmental composition by using autologous plasma. Therefore, pro-MMP9 measurements and correlations among lymphoid cell MMP9 content and secretion, mobility, invasiveness, and MD capacity were analyzed without cellular manipulations (such as transfection or exogenous chemo-attractive substance addition). Indeed, we observed a significant correlation between MMP9 content of freshly isolated cells and in vitro secretion (data not shown); in addition, a significant association between patients' lymphocytosis and in vitro invasiveness of lymphoid cells was demonstrated ($p = 0.03$, Figure 4). These relations seem to support the validity of our model in studying the mechanisms of Ly peripheral blood accumulation and extravasation in CLL.

In conclusion, B-CLL cells were shown to have a constitutively low migration capability, produce little MMP9, and be exposed to more higher levels of MMP9 of systemic origin. Plasma-free MMP9 levels were associated with blood cell accumulation, involving both leukemic and normal T/B cells, and had a prognostic value that could be useful in treatment decision making. Using, the whole lymphoid population in each patient together with his own plasma as an experimental model, it emerged that healthy Ly favor leukemic cell migration but in turn are inhibited in MMP9 production by increased CLL load. Moreover, endogenous plasma components affect cell mobility and invasiveness. Our data confirmed that the complex intertalk involving lymphoid cell subpopulations between themselves and systemic cytokines exerts an important role in CCL pathophysiology[20] and showed that it undergoes modifications over disease course. This observation deserves further investigation and may represent a key to define future tailoring strategies for anti-MMPs targeted treatments.[21]

Acknowledgements

We thank patients for contributing to this research and Dr Eros Ferrazzi for critical review. AIL (Associazione italiana leucemie e linfomi)-Rovigo local section and Regione Veneto (ricerca sanitaria finalizzata 2006) gave grants for the study. Milena Gusella, Caterina Bolzonella, Rossella Paolini, and Elisabetta Rodella initiated and designed the study; Rossella Paolini, Elisabetta Rodella, and Emilio Ramazzina recruited patients and performed clinical analysis; Caterina Bolzonella, Laura Bertolaso, Cinzia Scipioni, and Silvia Bellini realized biological studies; Milena Gusella, Antonio Cuneo, Emilio Ramazzina, and Felice Pasini participated in the data analysis; Milena Gusella, Felice Pasini, and Antonio Cuneo wrote the manuscript; and all authors critically reviewed the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

References

1. Jarvis SC, Snyderman R and Cohen HJ. Human lymphocyte motility: normal characteristics and anomalous behavior of chronic lymphocytic leukemia cells. *Blood* 1976; 48: 717–729.
2. Trinidad EM, Ballesteros M, Zuloaga J, et al. An impaired transendothelial migration potential of chronic lymphocytic leukemia (CLL) cells can be linked to ephrin-A4 expression. *Blood* 2009; 114: 5081–5090.
3. Redondo-Muñoz J, Escobar-Díaz E, Samaniego R, et al. MMP-9 in B-cell chronic lymphocytic leukemia is up-regulated by alpha4beta1 integrin or CXCR4 engagement via distinct signaling pathways, localizes to podosomes, and is involved in cell invasion and migration. *Blood* 2006; 108: 3143–3151.
4. Dufour A, Zucker S, Sampson NS, et al. Role of matrix metalloproteinase-9 dimers in cell migration: design of inhibitory peptides. *J Biol Chem* 2010; 285: 35944–35956.
5. Bauvois B, Dumont J, Mathiot C, et al. Production of matrix metalloproteinase-9 in early stage B-CLL: suppression by interferons. *Leukemia* 2002; 16: 791–798.
6. Kamiguti AS, Lee ES, Till KJ, et al. The role of matrix metalloproteinase 9 in the pathogenesis of chronic lymphocytic leukaemia. *Br J Haematol* 2004; 125: 128–140.
7. Redondo-Muñoz J, Ugart-Berzal E, García-Marco JA, et al. Alpha4beta1 integrin and 190-kDa CD44v constitute a cell surface docking complex for gelatinase B/MMP-9 in chronic leukemic but not in normal B cells. *Blood* 2008; 112: 169–178.
8. Bailón E, Ugarte-Berzal E, Amigo-Jiménez I, et al. Overexpression of progelatinase B/proMMP-9 affects migration regulatory pathways and impairs chronic lymphocytic leukemia cell homing to bone marrow and spleen. *J Leukoc Biol* 2014; 96: 185–199.
9. Redondo-Muñoz J, Ugarte-Berzal E, Terol MJ, et al. Matrix metalloproteinase-9 promotes chronic lymphocytic leukemia b cell survival through its hemopexin domain. *Cancer Cell* 2010; 17: 160–172.
10. Molica S, Vitelli G, Levato D, et al. Increased serum levels of matrix metalloproteinase-9 predict clinical outcome of patients with early B-cell chronic lymphocytic leukaemia. *Eur J Haematol* 2003; 70: 373–378.
11. Vaisitti T, Serra S, Pepper C, et al. CD38 signals upregulate expression and functions of matrix metalloproteinase-9 in chronic lymphocytic leukemia cells. *Leukemia* 2013; 27: 1177–1181.
12. Davids MS and Burger JA. Cell trafficking in chronic lymphocytic leukemia. *Open J Hematol* 2012; 3(S1): 3.
13. Chaudhary AK, Pandya S, Ghosh K, et al. Matrix metalloproteinase and its drug targets therapy in solid and hematological malignancies: an overview. *Mutat Res* 2013; 753: 7–23.
14. Vandooren J, Van den Steen PE and Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9): the next decade. *Crit Rev Biochem Mol Biol* 2013; 48: 222–272.
15. Kessenbrock K, Plaks V and Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* 2010; 141: 52–67.
16. Scrivener S, Goddard RV, Kaminski FR, et al. Abnormal T-cell function in B-cell chronic lymphocytic leukaemia. *Leuk Lymphoma* 2003; 44: 383–389.
17. Hock BD, Macpherson SA, Fernyhough LJ, et al. Chronic lymphocytic leukaemia cells become both activated and immunosuppressive following interaction with CD3 and CD28 stimulated PBMC. *Leuk Res* 2014; 38: 1217–1223.
18. Brusa D, Serra S, Coscia M, et al. The PD-1/PD-L1 axis contributes to T-cell dysfunction in chronic lymphocytic leukemia. *Haematologica* 2013; 98: 953–963.
19. Gassner FJ, Zaborsky N, Catakovic K, et al. Chronic lymphocytic leukaemia induces an exhausted T cell phenotype in the TCL1 transgenic mouse model. *Br J Haematol* 2015; 170: 515–522.
20. Shahjahani M, Mohammadiasl J, Noroozi F, et al. Molecular basis of chronic lymphocytic leukemia diagnosis and prognosis. *Cell Oncol* 2015; 38: 93–109.
21. ten Hacken E and Burger JA. Microenvironment dependency in Chronic Lymphocytic Leukemia: the basis for new targeted therapies. *Pharmacol Ther* 2014; 144: 338–348.