

Spontaneous and cytokine-evoked production of matrix metalloproteinases by bone marrow and peripheral blood pre-B cells in childhood acute lymphoblastic leukaemia

Rassa Pegahi, Florent Poyer, Elisabeth Legrand, Lionel Cazin, Jean-Pierre Vannier, Marek Lamacz

Groupe de recherche sur le Micro-Environnement et le Renouvellement Cellulaire Intégré M.E.R.C.I., Faculté de médecine-pharmacie, Université de Rouen, 22 bd Gambetta, 76183 Rouen Cedex, France

Correspondence : M. Lamacz <marek.lamacz@univ-rouen.fr>

Accepted for publication July 19, 2005

ABSTRACT. The present work focused on the study of the secretory activity of pre-B acute lymphoblastic leukaemia (ALL) cells harvested from bone marrow (BM) and peripheral blood (PB) in 16 children. The basal and cytokine (SDF-1, GM-CSF, bFGF, VEGF)-stimulated secretions of gelatinases 2 and 9 (MMPs-2 and -9) and expression of their genes were monitored by zymography and RT-PCR, respectively. A wide heterogeneity was found in the secretory capacities of these cells. The basal secretion of MMP-9 was more frequently observed than that of MMP-2 in both cell types. The cytokines VEGF and bFGF were found to induce predominant stimulatory effects on the MMP-2 secretion. In contrast, GM-CSF was shown to exert a more pronounced activation of the MMP-9 production. Experiments using inhibitors of metabolic pathways (U0126, LY294002 and SN50) revealed that the secretion of MMP-9 was mediated through PI3/MEK1 kinases. The MMP-2 secretion appeared to be however, stimulated through a different metabolic pathway. The microfluorimetric approach showed that the basal and stimulated secretions of MMPs-2 and -9 depended on the extracellular calcium pool. The cytokines VEGF and bFGF represent potent factors increasing the intracellular calcium concentration with similar kinetics. In contrast, GM-CSF was found to activate a verapamil-sensitive efflux of indo-1 from cytosol suggesting that this cytokine could be responsible for the activation of xenobiotic membrane transporters. Experiments using the trypan blue exclusion test demonstrated that bFGF, in contrast to VEGF and GM-CSF, markedly augmented pre-B ALL cell survival. Further investigations into a possible correlation between the plasma concentrations of MMP-2 and -9, VEGF, bFGF and GM-CSF, and the poor evolution of pre-B ALL in children could have valuable diagnostic implications.

Keywords: ALL cells, chemokines, bFGF, GM-CSF, VEGF, SDF-1, $[Ca^{2+}]_i$, PI3-kinase, MEK1

The acute lymphoblastic leukemia (ALL) is a childhood malignancy characterised by the presence of powerful hybrid molecules formed after translocation and fusion of an oncogene with a gene of another chromosome, allowing lymphoblasts to escape from the control of the cell cycle. The description of several different translocations has allowed a better understanding of the relatively high variability of the ALL cell properties that may directly govern the behavior of the cells and determine their resistance to chemical treatments. For these reasons, many ALL patients are exposed to relapse and fatal complications [1]. In some cases, the ALL symptoms may also be complicated by the invasive capacity of lymphoblasts, which have been found to preferentially colonize spleen, central nervous system, testis and/or liver [2].

The invasive capacities of solid cancers reflect their cellular ability to secrete different types of matrix metalloproteinases (MMPs) which, by inducing proteolytical collagen hydrolysis, are responsible for a reorganization of

the extracellular matrix facilitating metastases. Since MMP-2 and -9 (MMPs-2 and -9) are known to lead to cellular extravasation by degradation of native collagen IV and V forming the reticulation of the epithelial basal membrane, the secretion of gelatinases is considered as a worse prognosis in cancer evolution [3]. The MMPs-2 and -9, secreted as catalytically latent pro-enzymes, are activated either directly by several other peptidases or indirectly after initial formation of an intermediate complex with a tissue inhibitor of matrix metalloproteinases [3]. Several reports have revealed that expression of the genes coding for MMPs-2 and -9 both in normal and pathological cells may be mainly activated through three intracellular pathways including the NF κ B system, MEK1/2 or PI3 kinases [4-6]. More recently, Sun *et al.* (2002) showed that in synoviocytes, this activation would also be induced by a direct increase of the extracellular calcium concentration ($[Ca^{2+}]_e$). The suppression of this effect by an antagonist of MEK1/2 kinases [7] indicated that the enzymes involved

in the cascade of the MMPs-2 and -9 production are calcium-dependent and suggested that the transduction mechanisms required the recruitment of membrane calcium channels responsible for the extracellular calcium mobilization. In contrast, results obtained in keratinocytes, demonstrated that the activation of the extracellular signal-regulated kinases 1 and 2 (ERK 1/2) is responsible for an inhibition of the MMP secretion [8].

It is noteworthy that even if little is known about the mechanisms of MMPs-2 and -9 secretion in pre-T ALL cells, the production of MMP-9 could be considered as a possible marker of cellular invasiveness [9]. Thus, it should be pointed out that the characterization of the transduction mechanisms and determination of the role of calcium channels involved in the gelatinase secretion could contribute to the development of a strategy to block the MMP production in order to impede the cellular invasiveness. As normal lymphoblasts originate from the bone marrow and their differentiation continues in peripheral lymphopoietic organs, the evoked secretion of MMPs-2 and -9 by lymphoblasts in bone marrow may be considered as naturally necessary to the cell mobilization into the circulation. However, little is known about the constitutive secretory capacity of bone marrow (BM) and peripheral blood (PB) ALL cells. Since the environmental changes appear to be responsible for the determination of the cell phenotypes [10], it can be speculated that the PB lymphoblasts express a pathological proteolytic component conferring the invasive capacity. In order to elucidate these differences between BM and PB ALL cells, the constitutive and evoked secretions of gelatinases were studied and compared. The secretory capacities were evaluated by a zymography approach in the presence or absence of cytokines (VEGF, bFGF, GM-CSF and SDF-1) known to be involved in the control of the MMP secretion by solid tumors and chosen in the present study in order to investigate their possible role in the pathogenesis of ALL. The transduction mechanisms of these cytokines were investigated by analyzing the cytosolic calcium concentration and studying the effects of specific inhibitors of MEK1/2, PI3 kinase and NF κ B on MMPs-2 and -9 secretion. In addition, the expression of MMP genes was examined by RT-PCR allowing identification of MMP-2 and 9 mRNAs.

MATERIAL AND METHODS

Cells and conditioned media

Peripheral blood and bone marrow cell aliquots were collected from patients aged 1-16 years at presentation, or during their first or second relapse, for diagnosis procedures of ALL. The samples were collected with the informed consent of the children's parents, in agreement with the ethical laws of France. The diagnosis was established using conventional May Grünwald Giemsa and cytochemical stains in accordance with accepted FAB criteria, and confirmed by immunophenotyping using a panel of well-characterized monoclonal antibodies. The immunological subtypes pre-B (CD19+, CD10+, cytoplasmic immunoglobulin cIG-, and surface immunoglobulin were determined according to the GEIL criteria as previously

described [11]. In all cases, more than 85% of the leukemic cells were positive for CD19.

Leukemic samples were diluted with sterile RPMI-1640 medium and centrifuged on Ficoll-Hypaque (specific gravity: 1,077g/mL) (Eurobio, France). Washed in serum-free RPMI, mononuclear cells were used immediately for experiments, or frozen in liquid nitrogen after sedimentation and suspension in medium containing 10% DMSO and 10% foetal calf serum. The cells were suspended in serum-free Iscove at a final concentration of 2×10^6 cells/mL and incubated at 37°C in 5% CO₂ for 24 h in the presence or absence of cytokines.

Specific inhibitors of MEK1/2, PI3K and NF κ B, respectively U0126, LY294002, SN50 were used at 10 μ M. The cells were pre-incubated with inhibitors for 30 minutes before exposure to the cytokines bFGF (25ng/mL), VEGF (25ng/mL), GM-CSF (10ng/mL) and SDF-1 α (100 ng/mL). The concentrations of cytokines were previously determined in our laboratory in studies on ALL, haematopoietic CD34 and different cancer lineage cells. The measurements of MMP secretion were performed only once on a single sample from each patient tested.

The viability of the cells was assessed by trypan blue staining. The cell-conditioned medium (supernatants) was collected and analysed using zymography, or stored at -20°C. The cellular pellets were immediately lysed prior to extraction of their RNA or were stored at -80°C.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was extracted using an isolation kit (Promega) in accordance with the manufacturer's instructions. RNA concentration and purity were determined by measuring absorbance at 260 and 280 nm on an Ultraspec 3000 spectrophotometer (Pharmacia Biotech, Cambridge, England). Reverse transcription reactions (RT) were performed with approximately 1 μ g of total RNA heated to 94°C for 2 minutes. Polymerase chain reactions (PCR) were performed following the manufacturer's instructions (Promega). Reaction mixtures contained template DNA (RT), PCR reaction buffer, dNTP, and the desired upstream and down-stream primers.

Sequences for human MMP-2 and MMP-9 were obtained from GenBank (Los Alamos, NM). The following sequences of primers were used: i) MMP-2: 5'GGC CCT GTC ACT CCT GAG AT 3', 5' GGC ATC CAG GTT ATC GGG GA 3'; ii) MMP-9: 5'CCA CAT CAC CTA TTG GAT CC 3', 5'CGG GTG TAG AGT CTC TCG CT 3'. Each reaction mixture contained RT product (template DNA), the volume of which was determined by the amount necessary to equalise the intensities of actin bands visualised during agarose gel electrophoresis. For all primers, each reaction tube was incubated at 48°C for 45 minutes for RT. Thermocycling was performed with a Perkin Elmer Cetus 480 (USA) personal thermocycler at the optimum cycle number for each primer (35 for both gelatinases and 30 for actin). Each PCR cycle consisted of a heat denaturation step at 94°C for 1 min, a primer-annealing step at 60°C for 1 min, and a strand elongation step at 68°C for 1 min. Aliquots of PCR product (approximately 20 μ L)

were electrophoresed on 2% agarose gels containing 0.1 µg/mL ethidium bromide. Loading was equalized to the internal control mRNA (actin) to give equivalent signals. Gels were illuminated with UV light, photographed and/or digitized using an Image Analyser (Bio-Profil, Bioblock, France).

Gelatinase substrate zymography

Gelatinolytic activities in the cell-conditioned medium were analysed under non-reducing conditions using substrate (gelatin) sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (zymography) as described [12]. Samples mixed with loading buffer, were applied to a 10% polyacrylamide gel copolymerised with 2 mg/mL gelatin (Sigma), and electrophoresed at a constant voltage. The gels were washed in 2.5% Triton X100 (Sigma) for 1 hour to remove SDS and incubated in zymography buffer (50mM Tris/HCl, 5mM CaCl₂ pH 7.6) for 24 h at 37°C. Then, the gels were washed with unionised water and stained with 0.05% Coomassie Brilliant Blue G (Sigma) in 25% ethanol, 10% acetic acid water, and destained in 5% methanol, 7% acetic acid water. The gelatinase activities appeared as clear bands against a blue background. A serum 10% sample was used as positive control. The zymogrammes were processed using Image Processing and Analysis in Java (ImageJ, NIH Public domain).

Measurement of cytosolic calcium concentration

For microfluorimetric studies, the pre-B ALL cells were cultured on 25 mm diameter coverslips. The cells were incubated in the dark at 37°C for 30 min with 5 µM indo-1 dissolved in RPMI medium, supplemented with 15 mM HEPES. At the end of the incubation period, the cells were washed twice with 2 ml of fresh medium. The intracellular calcium concentration [Ca²⁺]_i was monitored by a dual-emission microfluorimeter system constructed from a Nikon Eclipse TE300 inverted microscope, as previously described [13]. The fluorescence emission of indo-1, induced by excitation at 355 nm, was recorded at two wavelengths (405 and 480 nm) by separate photometers (Nikon). Both signals were continuously recorded by PC, and the 405/480 ratio (R), illustrating calcium variations, was obtained using an algorithm developed by Notocord Systems (Croissy sur Seine, France). The actual values for [Ca²⁺]_i were calculated from the following formula established by Grynkiewicz *et al.* (1985) [Ca²⁺]_i = Kd x β(R-R_{min})/(R_{max}-R) [14] where R is the 405/480 nm ratio, R_{min} represents the minimum fluorescence ratio obtained after the incubation of cells with 10 mM EGTA and 10 µM ionomycin for 3 h, R_{max} represents the minimum fluorescence ratio obtained after the incubation of cells with 10 mM CaCl₂ and 10 µM ionomycin for 3h, and β is the ratio of fluorescence yield from the Ca²⁺_{min}/Ca²⁺_{max} indicator at 480 nm. The average values of R_{min}, R_{max}, and β were 0.14, 2.02 and 1.59 respectively. Kd = 250 nM is the dissociation constant for indo-1 AM [14]. Cytokines were injected in the vicinity of the cells by a pressure ejected system constructed by M.L.

RESULTS

Basal and cytokine-induced MMPs-2 and -9 secretion in cultured pre-B ALL cells

The capacity of cultured primary ALL cells to secrete MMPs-2 and -9 into culture medium was monitored by gelatine substrate zymography after a 24 h period of incubation.

Figure 1A illustrates a representative gel obtained in the presence or absence of different cytokines and inhibitors of metabolic pathways tested herein. The density values of the enzymatic bands (expressed as arbitrary units) measured under different experimental conditions allowed us to compare the levels of basal and cytokine-evoked secretions (figure 1B, C). Altogether, the samples of PB (n = 9) and BM (n = 9) cells tested were obtained from 16 patients. Among these samples, only two of them originated from same patient. The cells were studied after thawing apart from two samples of PB cells and two samples of BM cells that were used just after isolation.

Both PB and BM cells showed a great heterogeneity in their constitutive secretory activities. From the secretory patterns observed in these samples, three groups of cells could be distinguished: i) secreting cells producing both MMP-2 and MMP-9 (PB: n = 2; BM n = 4, ii) secreting cells producing either MMP-9 (PB n = 4; BM n = 2) or MMP-2 (PB n = 0; BM n = 1) and iii) non-secreting cells (PB n = 3; BM n = 1).

The VEGF appeared to be able to stimulate the production of MMP-2 in both cell types. In contrast, its effect on MMP-9 secretion was often undetectable or very modest. When compared to the VEGF responses, the effects of bFGF on MMP-2 secretion appeared to be more frequent and pronounced. Similarly, as observed with VEGF, effects of bFGF on MMP-9 secretion were detected less frequently but with higher amplitude in BM cells.

In experiments using GM-CSF, it was found that this cytokine failed to modify the basal level of MMP-2 secretion in both PB and BM cells, except in one sample of BM cells in which the secretion was enhanced. In contrast, in five out of nine samples of PB cells, the MMP-9 secretion appeared to have been stimulated.

In SDF1-incubated PB and BM cells no marked change in the levels of secretion of either enzyme was detected.

It must be mentioned that in the samples exhibiting a high level of spontaneous secretion, the responsiveness to cytokines was noticeably weaker or absent.

MMP-2 and MMP-9 gene expression in pre-B ALL cells

Figure 2 shows representative RT-PCR bands corresponding to mRNAs of both MMP-9 and MMP-2. In cells that did not exhibit any constitutive secretion of MMP-2 or -9, the mRNAs corresponding to these enzymes failed to be detected. In a good agreement with the cytokine-induced enzyme secretion described above, addition of cytokines rendered the level of mRNAs detectable (figure 2A, B). In contrast, in cells exhibiting a basal secretory activity, mRNAs were found and their levels were markedly increased or decreased by cytokines or metabolic pathway inhibitors, respectively (figure 2A).

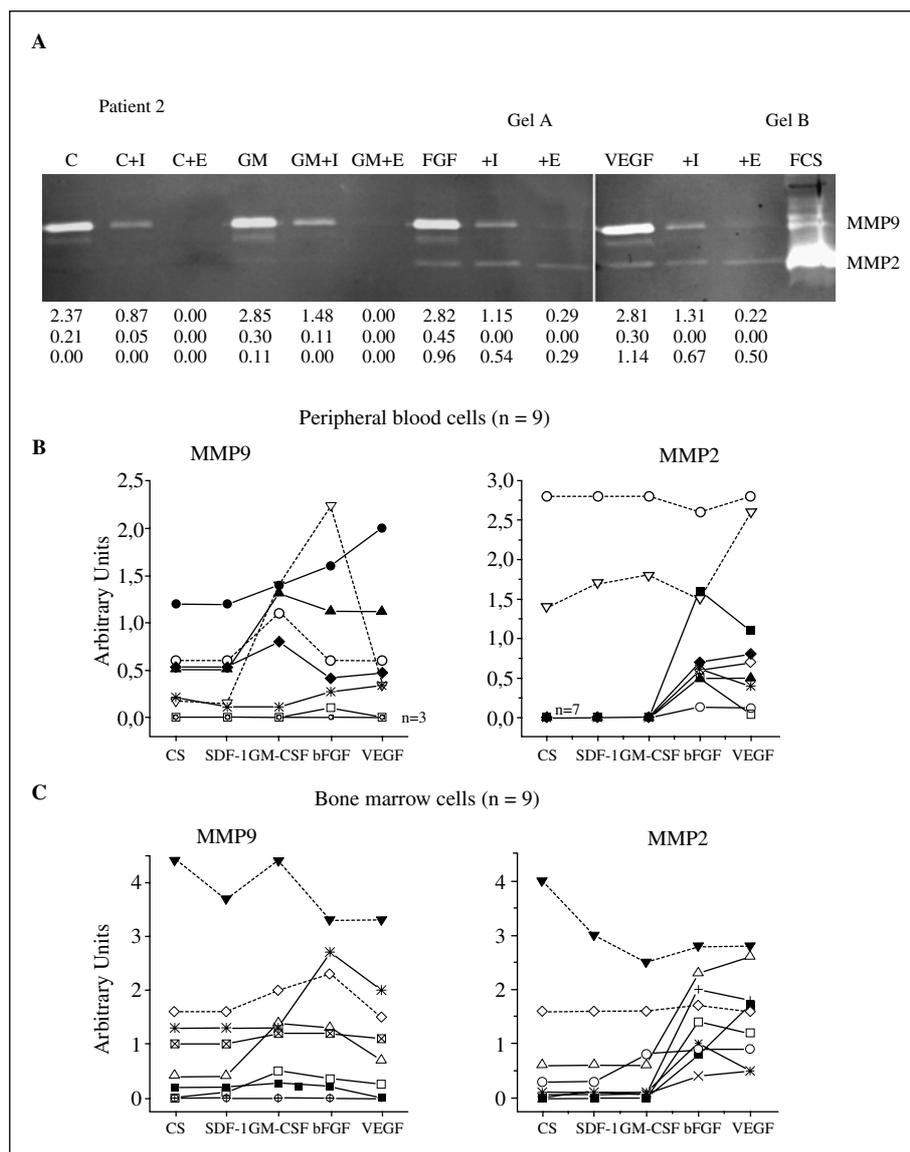


Figure 1

Constitutive and cytokine-induced secretion of MMPs-2 and -9 by PB and BM pre-B ALL cells. **A**) Representative zymogram obtained during analysis of gelatinases contained in serum-free ISCOVE media after a 24-h incubation of PB ALL cells in the absence (c) or presence of cytokines (GM: granulocyte-monocyte colony stimulating factor; FGF: b fibroblast growth factor; VEGF – vascular endothelial growth factor); I: U0126, a MEK1/2 inhibitor; E: EDTA, fetal calf serum (FCS) was used as standard, showing the positions of the 92 kDa (MMP-9) and 72 kDa (MMP-2) activities in the gel. The density of each band is represented as arbitrary units placed below the zymogram respectively. **B** and **C**) Resulting curves of constitutive and cytokine-stimulated secretions of MMP-2 (right) and MMP-9 (left) in PB (**B**) and BM (**C**) pre-B ALL cells. Each curve corresponds to enzymatic activities detected in the culture medium from one patient. Dotted and solid lines represent data obtained from fresh and thawed cells respectively.

Effects of metabolic pathway inhibitors and a free-calcium depressor on MMPs-2 and -9 secretions

In order to characterize the intracellular mechanisms of MMPs-2 and -9 secretion, three antagonists of intracellular metabolic pathways, namely U0126, LY 294002 and SN50, were used to block MEK1/2, PI3K and NF κ B, respectively [4-6]. The calcium-dependency of the enzyme secretion was also investigated, by lowering the extracellular free calcium concentration with EDTA, added to the culture medium. In addition, the capacity of the cytokines studied to increase the cytosolic calcium concentration was tested using the microfluorimetric approach.

The comparison of the effects of the inhibitors tested on the MMPs-2 and -9 production by PB and BM cells is illustrated

by the histograms shown in *figure 3*. U0126, an antagonist of the MEK-1 kinase pathway, markedly decreased the basal and stimulated secretions of MMP-9 in PB cells. In BM cells, its inhibitory effect appeared to be less pronounced. In contrast, this antagonist was found to exert only slight inhibitory effects on the MMP-2 secretion.

LY 294002, an inhibitor of PI3K, diminished by $\approx 30\%$ the stimulatory effects on the MMPs-2 and -9 secretions provoked by the all cytokines tested (*figure 3*). Its effect on the cytokine-induced MMP-9 production by BM cells appeared to be more efficient in the VEGF-evoked enzyme secretion.

When compared to the other inhibitors used in the present study, the SN50 peptide, an NF κ B inhibitor, provoked a less pronounced decrease in MMPs-2 and -9 secretion.

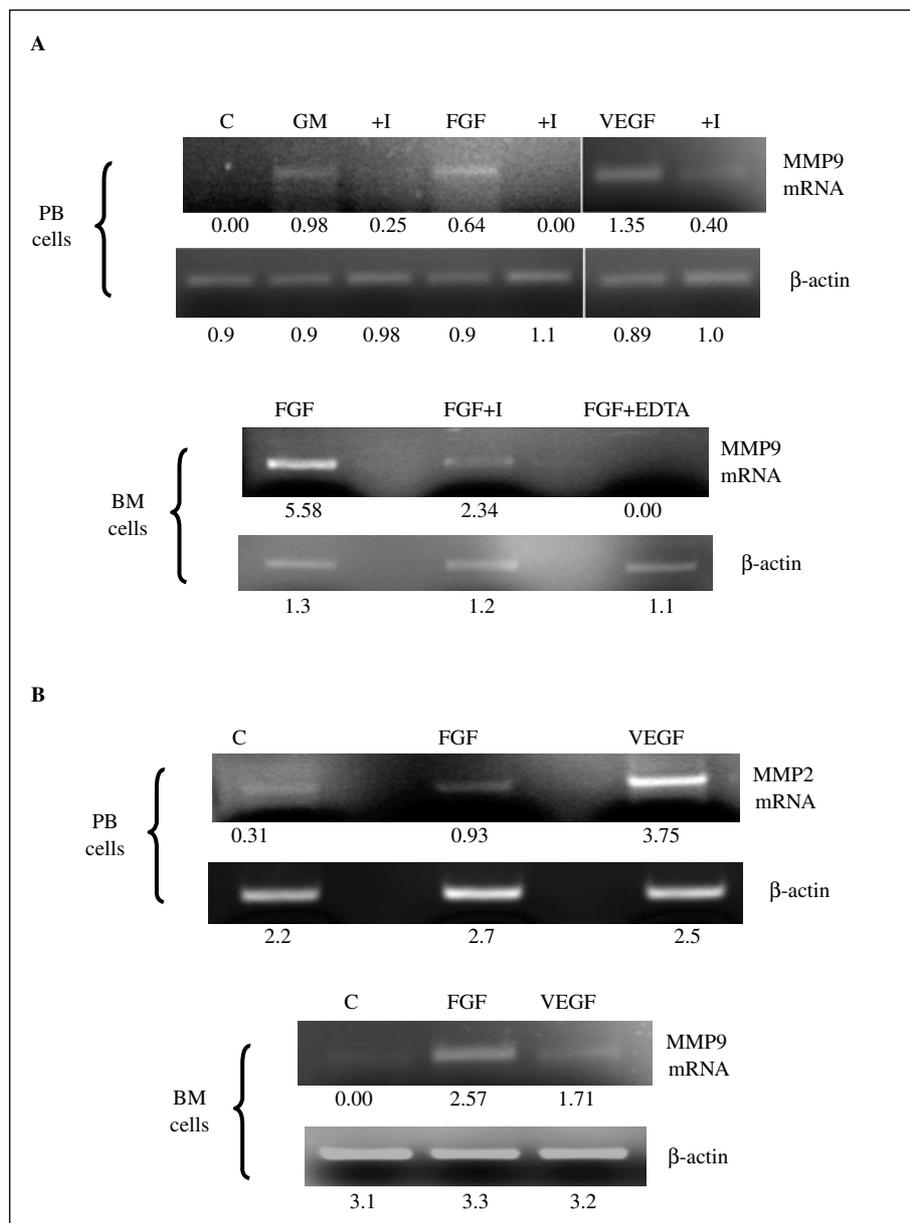


Figure 2

RT-PCR analysis of pro-MMPs-2 and -9 mRNA extracted from pre-B ALL cells. **A)** Representative example of cDNA detection templating on MMP-9 mRNA respectively produced by PB and BM cells incubated in the absence (c: control) or presence of cytokines and U0126 (*figure 1A*). **B)** RT-PCR analysis of MMP-2 mRNA transcripts in PB and BM pre-B ALL cells incubated in the absence (c: control) or presence of bFGF and VEGF. In **A** and **B**, β -actin was used as the internal control.

In contrast to the data -described above, extracellular application of EDTA markedly depressed both the spontaneous and cytokine-stimulated secretions of MMPs-2 and -9 (*figure 3*). In parallel to these findings, as shown in *figure 4*, microfluorimetric measurements of $[Ca^{2+}]_i$ performed in single ALL cells revealed that the cytokines markedly influenced the cytosolic calcium level. Extracellular application of KCl (35 mM) or Ca^{2+} (10 mM) provoked in PB, as well as in BM cells, a pronounced, transient increase in $[Ca^{2+}]_i$, reaching 200 nM before recovering the basal level within 100 sec. In a good agreement with these observations, the addition of EDTA to the culture medium dramatically decreased the basal cytosolic calcium level (data not shown).

Another set of experiments was undertaken to determine whether the cytokines tested were able to modify calcium

homeostasis in pre-B ALL cells. All the cytokines tested, apart from SDF, changed the cytosolic calcium concentration. As observed from the representative examples shown in *figure 4B, C*, bFGF and VEGF strongly increased $[Ca^{2+}]_i$ with similar time-courses. Surprisingly, GM-CSF provoked a decrease in fluorescence emission of both 405 and 480 nm spectra, suggesting the existence of either a quenching of fluorescent signals by another population of bivalent ions or the disappearance of indo-1 from the cytosol.

All of these effects of bFGF and VEGF were found to be abolished by the addition of EDTA to the extracellular medium. Moreover, EDTA and verapamil impeded the double disappearance of fluorescent signals, suggesting that the GM-CSF-evoked effects depended on calcium influx (data not shown).

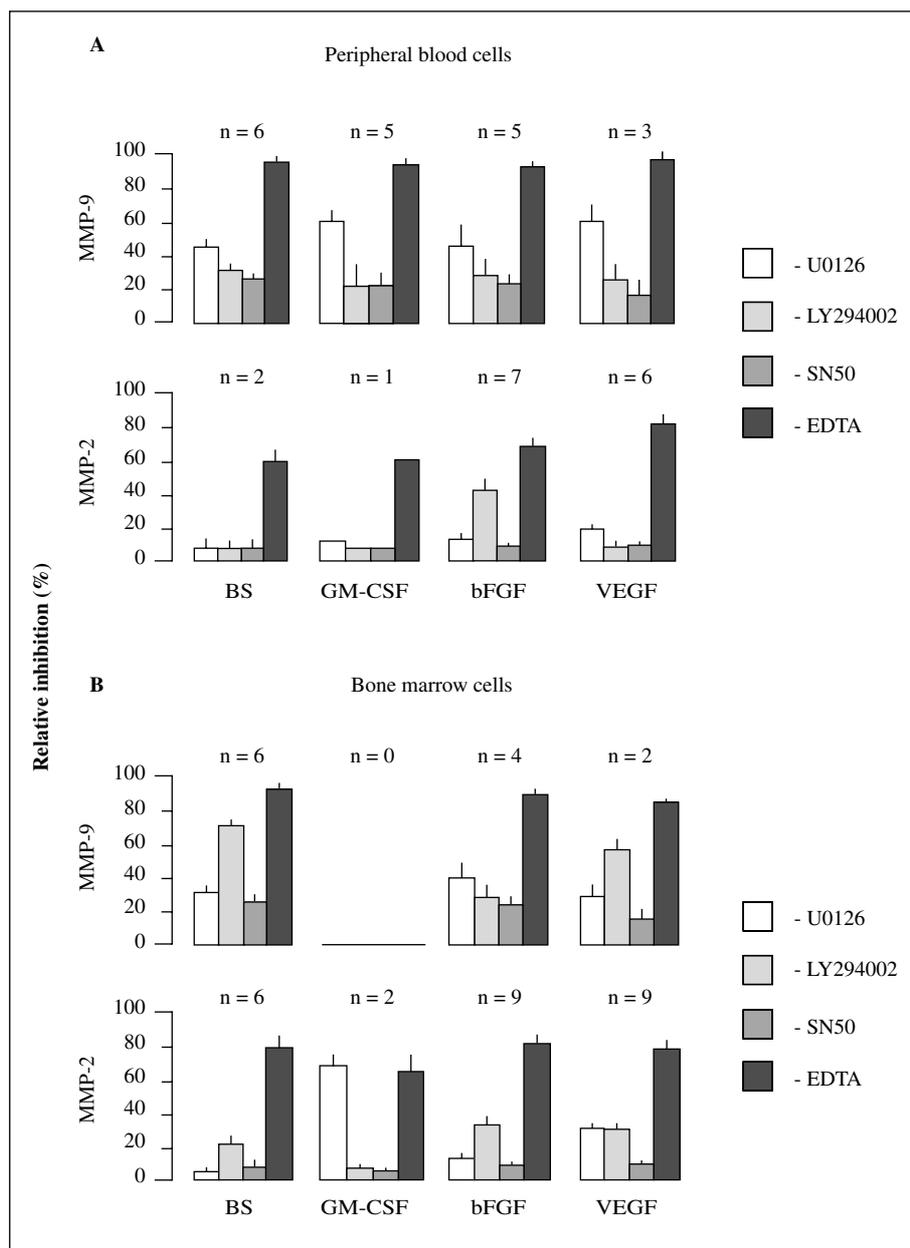


Figure 3

Effects of metabolic pathway inhibitors on the basal and cytokine-stimulated secretion of MMPs-2 and -9. The histograms represent the percentages of relative inhibition expressed from the basal or cytokine-stimulated secretion level (\pm SEM) of MMP-9 or MMP-2 in PB (A) and BM (B) pre-B ALL cells in the presence of metabolic pathway inhibitors. Each bar corresponds to the inhibition provoked by U0126, LY 294002, SN50 or EDTA respectively.

Cytokine-dependent viability of ALL cultured cells

The survival of leukemia cells, which is known to be more limited in the peripheral circulation than in bone marrow [15, 16], was found to be increased by the presence of certain cytokines and growth factors [17]. Thus, the protective role of the cytokines tested in the present study was investigated using a Trypan blue exclusion test.

As shown in figure 5, after a 60-h incubation with VEGF and GM-CSF, the percentage of cellular viability ($\approx 40\%$) did not significantly differ ($p < 0.9$) from the control. In contrast, incubation with bFGF markedly increased the percentage of cells surviving. After a 60-h incubation, this percentage exceeded 87% and differed significantly from the control ($p < 0.001$, Student's *t*-test).

DISCUSSION

In solid cancers, the secretion of MMPs-2 and -9 is considered to be a marker of a poor prognosis in the evolution of the disease [3]. This was based on findings suggesting that degradation by gelatinases of the interstitial reticulation composed of IV and V collagens facilitates cell migration, conditioning their aggressiveness and metastasis. The same hypothesis was proposed to explain an extramedullary infiltration by certain disseminated cancers such as myeloma or chronic lymphoblastic leukaemia [9, 18]. However, so far little is known about a possible role of MMPs in the invasiveness of pre-B ALL cells.

The present work revealed the existence of a constitutive secretion of MMPs-2 and -9 in both PB and BM cells.

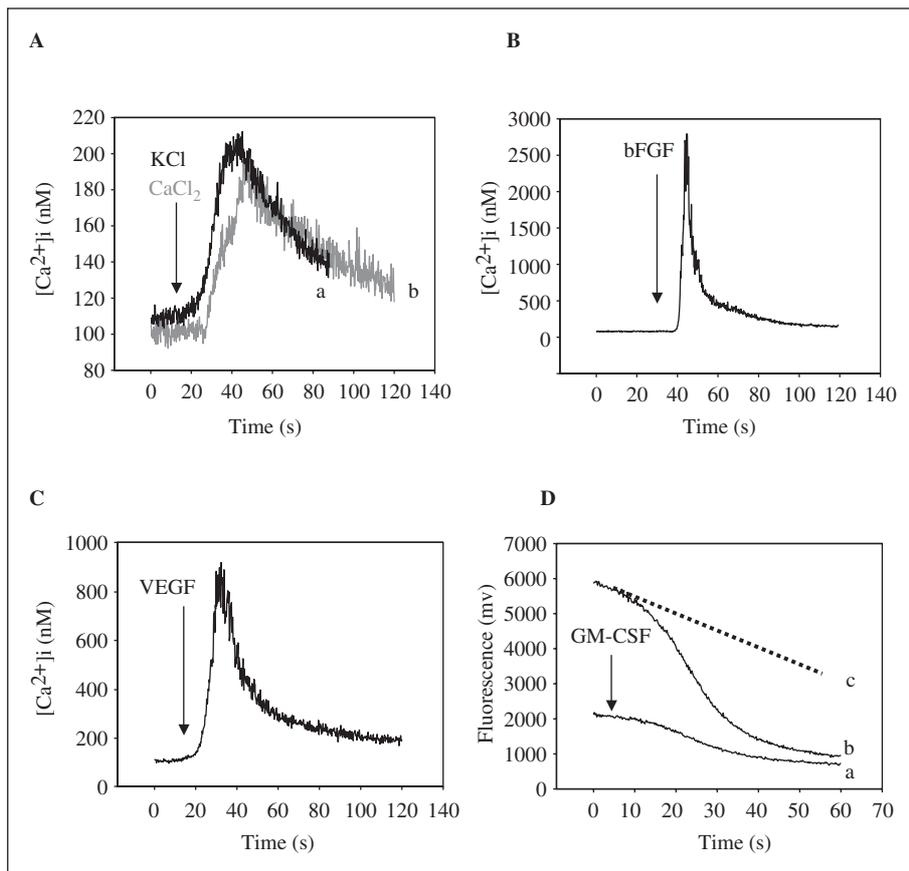


Figure 4

Effects of KCl, CaCl₂ and cytokines on [Ca²⁺]_i in pre-B ALL cells. **A**) Representative superimposed microfluorimetric recordings of [Ca²⁺]_i performed from two single cells. The pulses of KCl (50 mM, 15 s, a) and CaCl₂ (24 mM, 15 s, b) were administered in the vicinity of the cells. **B, C**) Microfluorimetric recordings obtained from two other cells in response to bFGF (20 ng/ml, 10 s, B) or VEGF (20 ng/ml, 10 s, C). **D**) Effect of GM-CSF (20 ng/ml, 15 s) on the evolution of 405 (a) and 480 nm (b) fluorescent signals. The curve c shows the natural depletion of the 480 nm signal due to photo-bleaching. The arrows indicate the onset of cytokine administration. The experiments were performed in a total of 20 (Aa), 15 (Ab), 30 (B), 28 (C) and 40 cells (D).

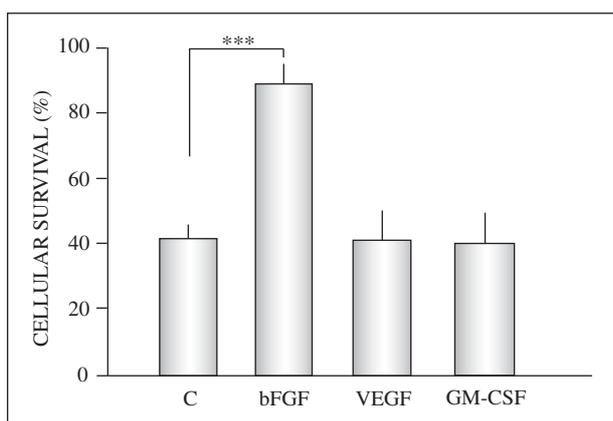


Figure 5

Percentages of cell survival in cultures of pre-B ALL cells in the absence (control: C) or presence of bFGF, GM-CSF and VEGF. After a 60-h period of culture, the cells were incubated for 15 minutes in trypan blue-containing medium. The viable, non-stained cells were expressed as a percentage relative to a total number of 300 cells counted in each test. The data represent mean ± SEM of four independent experiments.

Interestingly, the spontaneous secretion of MMP-9 appeared to be more pronounced in both PB and BM cells. It was observed that, as in bladder cancer cells [19], 50% of pre-B ALL cells were able to spontaneously produce these

gelatinases (figure 1). It should be mentioned, that if normal PB lymphoblasts can spontaneously secrete MMPs-2 and -9, those isolated from BM cells appeared to be devoid of this capacity [20]. Knowing that the vast majority of human pre-B ALL cells are CD34+ positive [21], the constitutive production of gelatinases deduced from the present findings, may be considered as a change from a normal to a tumour state. The very great heterogeneity of enzymatic activity observed in basal (as in stimulated) conditions could be explained by the several chromosomal aberrations known to trigger leukaemia [1].

Our investigations also focused on the control of the secretory activities of MMPs-2 and -9 by different cytokines recognized as playing a crucial role in the evolution of solid cancers [3, 22-25]. MMPs-2 and -9 secretions were found to be controlled with varying efficiency by cytokines such as VEGF, bFGF, GM-CSF or SDF-1.

The chemokine SDF-1 and its specific receptor CXCR4, are known to be involved in the trafficking of hematopoietic and cancer cells [26, 27]. Thus, the expected effect of SDF-1 on MMPs-2 and -9 mobilization was not observed. However, it must be mentioned that in a previous work, we observed a high density of CXCR4 that only weakly correlated with the cellular migration [26]. Altogether, these observations appear to contradict those obtained by Crazzolaro *et al.* (2001) who reported that a high number of

CXCR4 in ALL cells determined their capacity to infiltrate extra-medullary organs. This infiltration would result from the SDF-1-induced increase in cellular mobility and extravasation that are facilitated by MMPs-2 and -9 [2]. Since the degradation of CXCR4 by protease activities was found in Jurkat lymphoblastoid T cells [28], it can be speculated that these activities increasing within the culture medium over a period of several hours, may be responsible for the CXCR4 proteolysis and a loss of its biological activity. In a good agreement with this, it was also observed in the present study that SDF-1 did not modify the calcium mobilization.

In contrast to the SDF-1-induced effects, the stimulation provoked by VEGF and bFGF on MMPs-2 and -9 secretion, was markedly pronounced and more particularly, in cells exhibiting a weak, spontaneous secretion of gelatinases. Since, the gelatinases are considered to be markers of poor prognosis in leukaemia, it may be suggested that the cytokines VEGF and bFGF, which appeared to be stimulators of MMPs-2 and -9, could represent complementary markers. In a previous work, Schneider *et al.* (2003) reported that in ALL patients, the urinary concentration of bFGF was lower while the plasma level of VEGF was higher than in normal subjects [29]. In addition, Yetgin *et al.* (2001) demonstrated that remission of the disease is marked by a decrease in plasma concentration of bFGF associated with an increase in VEGF [30]. It was also reported that myeloid leukaemia is characterized by an increase in bFGF and VEGF concentrations, while ALL patients were found to exhibit a high level of bFGF and low level of VEGF [16]. Together with these findings, the results of the present work showing the existence of both a consistent, constitutive secretion of MMP-9 and the cytokine-activated secretion of MMP-2, strongly suggest that measurements of the plasma levels of MMPs-2 and -9 and the cytokines bFGF and VEGF should give a better appreciation of the prognosis for ALL.

Our investigations using GM-CSF revealed that this cytokine was only able to enhance the MMP-9 secretion in PB and BM cells. This ability of GM-CSF to control the activity of pre-B ALL cells represents an original finding, demonstrating once again that the effect of this cytokine is not exclusively confined to myeloid cells. So far, it has been shown that chronic lymphoblastic leukaemia cells may express two chains of the GM-CSF receptor [31]. As recently reported, GM-CSF may enforce conversion of pro-T cells for myelomonocytic differentiation [32], which helps explain the previously described lineage infidelity of ALL cells differentiated in AML cells [33]. The control of GM-CSF over gelatinase activity in the different types of malignancies is not as well documented as that exerted by bFGF or VEGF. Nevertheless, an autocrine mechanism for the GM-CSF-stimulated production of gelatinases and urokinase plasminogen activator was found to be responsible for invasion by lung cancer cells [34].

Another question raised in this study concerns the ionic and molecular mechanisms involved in the secretion of MMPs-2 and -9 by pre-B ALL cells. The constitutive and cytokine-evoked production of MMPs-2 and -9 is stimulated by a cascade of metabolic pathways recruiting mostly AP-1 and NF κ B proteins that are indispensable for the activation of gene promoters [35, 36]. Phosphorylation of these transcription factors may vary with cell types. In hepatocellular carcinoma, the mobilization of the MMP-9

gene depends on ERK and PI3K/AKT pathways respectively [37]. In addition, the involvement of c-Jun N-terminal kinase, p38, PKC and Ras pathway was observed in other cell types [38]. In a recent study on B-cell chronic lymphatic leukaemia, it was shown that PI3K/PKC and p38 MAPK pathways were implicated in the constitutive secretion of MMP-9 [39].

Our results obtained with U0126 and LY 294002, ERK and PI3K inhibitors, suggest that the secretion of MMP-9 in pre-B ALL cells could depend on ERK1/2 and PI3K activities. The weak inhibitory effect of the SN50 peptide observed in the present study may be explained by the fact that the inhibition of only one transcription factor is not sufficient to block MMP-9 production. Moreover, as recently observed in HT 1080 cells, the inhibitory effect of SN50 is not always statistically significant and examination of the expression of transfected NF κ B reporter vector gave more convincing results [35].

The stimulation of MMP-2 also depends on NF κ B. The activity of this protein may be stimulated by PI3K or COX-2 and tyrosine kinase transduction in HT 1080 fibrosarcoma cell line [35] and normal human pulp cells [40] respectively.

Our observations that the inhibitors used in the present experiments were not very efficient as regards the production of MMP-2 in pre-B ALL cells, suggests the implication of another, as yet undetermined metabolic pathway in the secretion of this enzyme.

The question of whether calcium ions play a role in the production and release of MMPs-2 and -9 in pre-B ALL cells was addressed using EDTA. It appeared that extracellular administration of EDTA dramatically depressed the secretion of the gelatinases. Moreover, the RT-PCR approach revealed that mRNA production also vanished in the presence of EDTA. Altogether, these results demonstrate, for the first time, that in pre-B ALL cells both exocytosis and gene activation depend on extracellular calcium. Similar results were found in synoviocytes in which an increase in the extracellular calcium concentration activated the induction of MMP1 promoter [7].

In the present work, it was also observed for the first time that in ALL single cells, calcium homeostasis depended on voltage-activated calcium channels. This was based on the observation that extracellular administration of KCl at a depolarizing concentration, or CaCl₂ at a high concentration, provoked an increase in the cytosolic calcium ions. Another original finding was that VEGF, bFGF and GM-CSF appeared to be able to mobilize cytosolic calcium ions. Both VEGF and bFGF were powerful stimulators of cytosolic calcium mobilization. The similar kinetics found associated with effects did not exclude the idea that they could act through different transduction mechanisms. As a matter of fact, from recent studies carried out in choroidal endothelial cells it was speculated that VEGF can increase the cytosolic calcium concentration through a PLC pathway [41, 42] while the bFGF-induced action observed in cardiac myocytes would more likely result from the activation of voltage-dependent, membrane calcium channels [43, 44].

The results obtained with GM-CSF differed markedly from those described above, with the disappearance of both fluorescence signals occurring. This phenomenon could not result from a photo-bleaching of indo-1 since, in this hypothesis, a decrease in fluorescence would have

followed the initial slope of the curve. In fact, the abrupt breaking down of the slope observed after each injection of GM-CSF reflected a decrease in the cytosolic indo-1 concentration, very likely caused by a dye efflux. This event could be explained by the activity of ATP-binding cassette transporters, known as multidrug-resistance proteins (MRP) shown to be abundant in normal and malignant hematopoietic progenitors [45]. As reported in CD34⁺/38⁻ cells, the ATP-dependent, MRP-mediated efflux of the two fluorescent substrates, rhodamine-123 and acetoxymethyl ester of calcein, was blocked by verapamil, which is also a very well-known L-type calcium channel blocker. Since GM-CSF failed to decrease the intensity of the fluorescence signals in the presence of verapamil, it was concluded that the activation of MRP could be calcium-dependent. Altogether, these findings strongly suggest that GM-CSF could be considered as a factor of chemoresistance. Thus, the question of whether a high plasma level of this cytokine has a diagnostic value that could be used in the development of chemotherapy protocols for ALL patients should be addressed.

The role of the cytokines in the survival of pre-B ALL cells was examined in experiments using the trypan blue exclusion test. It was demonstrated that, among the three cytokines tested, bFGF was the only one to be able to enhance the duration of cell survival. Similar effects were previously described in different normal cell types including epithelial cells, smooth muscle and neurons [46]. These effects were found to result from the inhibition of Ca-dependent kinases following a loss of internal calcium through Ca-dependent ATPases. Alternatively, bFGF was also found to be able to induce an up-regulation process of anti-apoptotic Bcl-2 protein known to protect MCF-7 breast cancer cells [47]. According to these observations, it can be speculated that high bFGF plasma levels may impede apoptosis in PB ALL cells.

In conclusion, the data generated by the present study reveal a large heterogeneity in constitutive and cytokine-evoked secretions of MMPs-2 and -9 in pre-B ALL cells. These secretions closely depend on extracellular calcium. MMPs-2 and -9 secretions appeared to have different activation pathways. The secretion of MMP-9 was mediated through PI3/MEK1 kinases, while MMP-2 production seemed to be stimulated through a different metabolic pathway. The cytokines bFGF and VEGF represent potent factors that increase the intracellular calcium concentration. The cytokine GM-CSF may provoke the activation of xenobiotic membrane transporters. Further clinical studies are required to evaluate the capacity of bFGF and GM-CSF to increase cell survival and chemoresistance respectively, which could be correlated with a poor evolution of ALL disease in children.

Acknowledgments. We thank the laboratory assistants of the Haematology Laboratory of CHU of Rouen for their technical assistance. This work was supported by a Research Grant from the GEFLUC of Haute-Normandie and the Ligue Contre le Cancer of Seine-Maritime.

REFERENCES

1. Hoelzer DN, Gokbuget O, Ottmann CH, *et al.* Acute lymphoblastic leukemia. *Hematology (Am Soc Hematol Educ Program)* 2002; 162.
2. Crazzolara R, Kreczy A, Mann G, *et al.* High expression of the chemokine receptor CXCR4 predicts extramedullary organ infiltration in childhood acute lymphoblastic leukaemia. *Br J Haematol* 2001; 115: 545.
3. Foda HD, Zucker S. Matrix metalloproteinases in cancer invasion, metastasis and angiogenesis. *Drug Discov Today* 2001; 6: 478.
4. Oshiro T, Shibasaki Y, Martin TJ, *et al.* Immunolocalization of vacuolar-type H⁺-ATPase, cathepsin K, matrix metalloproteinase-9, and receptor activator of NFκB ligand in odontoclasts during physiological root resorption of human deciduous teeth. *Anat Rec* 2001; 264: 305.
5. Welch DR, Sakamaki T, Pioquinto R, *et al.* Transfection of constitutively active mitogen-activated protein/extracellular signal-regulated kinase kinase confers tumorigenic and metastatic potentials to NIH3T3 cells. *Cancer Res* 2000; 60: 1552.
6. Liao X, Thrasher JB, Pelling J, *et al.* Androgen stimulates matrix metalloproteinase-2 expression in human prostate cancer. *Endocrinology* 2003; 144: 1656.
7. Sun Y, Wenger L, Brinckerhoff CE, *et al.* Basic calcium phosphate crystals induce matrix metalloproteinase-1 through the Ras/mitogen-activated protein kinase/c-Fos/AP-1/metalloproteinase 1 pathway. Involvement of transcription factor binding sites AP-1 and PEA-3. *J Biol Chem* 2002; 277: 1544.
8. Ala-aho R, Johansson N, Grenman R, *et al.* Inhibition of collagenase-3 (MMP-13) expression in transformed human keratinocytes by interferon-gamma is associated with activation of extracellular signal-regulated kinase-1,2 and STAT1. *Oncogene* 2000; 19: 248.
9. Hayashibara T, Yamada Y, Onimaru Y, *et al.* Matrix metalloproteinase-9 and vascular endothelial growth factor: a possible link in adult T-cell leukaemia cell invasion. *Br J Haematol* 2002; 116: 94.
10. Steidl U, Kronenwett R, Rohr UP, *et al.* Gene expression profiling identifies significant differences between the molecular phenotypes of bone marrow-derived and circulating human CD34⁺ hematopoietic stem cells. *Blood* 2002; 99: 2037.
11. Lenormand B, Bene MC, Lesesve JF, *et al.* PreB1 (CD10⁻) acute lymphoblastic leukemia: immunophenotypic and genomic characteristics, clinical features and outcome in 38 adults and 26 children. The Groupe d'Etude Immunologique des Leucemies. *Leuk Lymphoma* 1998; 28: 329.
12. Stetler-Stevenson WG. Progelatinase A activation during tumor cell invasion. *Invasion Metastasis* 1994; 14: 259.
13. Galas L, Garnier M, Lamacz M. Calcium waves in frog melanotrophs are generated by intracellular inactivation of TTX-sensitive membrane Na⁺ channel. *Mol Cell Endocrinol* 2000; 170: 197.
14. Gryniewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; 260: 3440.
15. Testa NG, Dexter TM. Long-term hematopoietic damage: concepts, approaches, and results relevant to the study of environmental toxins. *Environ Health Perspect* 1989; 82: 51.
16. Aguayo A, Kantarjian H, Manshour T, *et al.* Angiogenesis in acute and chronic leukemias and myelodysplastic syndromes. *Blood* 2000; 96: 2240.
17. Fujimura S, Arakawa F, Yamada Y, *et al.* Growth arrest and apoptosis in adult T cell leukemia cell lines following IL-2 deprivation. *Int J Oncol* 2004; 25: 437.
18. Klein G, Vellenga E, Fraaije MW, *et al.* The possible role of matrix metalloproteinase (MMP)-2 and MMP-9 in cancer, e.g. acute leukemia. *Crit Rev Oncol Hematol* 2004; 50: 87.
19. Vasala K, Paakko P, Turpeenniemi-Hujanen T. Matrix metalloproteinase-2 immunoreactive protein as a prognostic marker in bladder cancer. *Urology* 2003; 62: 952.

20. Janowska-Wieczorek A, Marquez LA, Nabholz JM, *et al.* Growth factors and cytokines upregulate gelatinase expression in bone marrow CD34(+) cells and their transmigration through reconstituted basement membrane. *Blood* 1999; 93: 3379.
21. Ko BS, Tang JL, Tsai W, *et al.* Philadelphia chromosome-positive acute lymphoblastic leukemia in Taiwan. *Ann Hematol* 2001; 80: 510.
22. Herrera CA, Xu L, Bucana CD, *et al.* Expression of metastasis-related genes in human epithelial ovarian tumors. *Int J Oncol* 2002; 20: 5.
23. Billottet C, Janji B, Thiery JP, *et al.* Rapid tumor development and potent vascularization are independent events in carcinoma producing FGF-1 or FGF-2. *Oncogene* 2002; 21: 8128.
24. Pei X, Nakanishi HY, Takayama K, *et al.* Granulocyte, granulocyte-macrophage, and macrophage colony-stimulating factors can stimulate the invasive capacity of human lung cancer cells. *Br J Cancer* 1999; 79: 40.
25. Yoneda J, Kuniyasu H, Crispens MA, *et al.* Expression of angiogenesis-related genes and progression of human ovarian carcinomas in nude mice. *J Natl Cancer Inst* 1998; 90: 447.
26. Sbaa-Ketata E, Vasse M, Lenormand B, *et al.* Fibronectin increases the migration induced by stromal cell-derived factor-1 alpha (SDF-1 alpha) in pre-B acute lymphoblastic leukemia cells. *Eur Cytokine Netw* 2001; 12: 223.
27. Muller AB, Homey H, Soto N, *et al.* Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001; 410: 50.
28. Valenzuela-Fernandez A, Planchenault T, Baleux F, *et al.* Leukocyte elastase negatively regulates Stromal cell-derived factor-1 (SDF-1)/CXCR4 binding and functions by amino-terminal processing of SDF-1 and CXCR4. *J Biol Chem* 2002; 277: 15677.
29. Schneider P, Vasse M, Legrand E, *et al.* Have urinary levels of the angiogenic factors, basic fibroblast growth factor and vascular endothelial growth factor, a prognostic value in childhood acute lymphoblastic leukaemia? *Br J Haematol* 2003; 122: 163.
30. Yetgin S, Yenicesu I, Cetin M, *et al.* Clinical importance of serum vascular endothelial and basic fibroblast growth factors in children with acute lymphoblastic leukemia. *Leuk Lymphoma* 2001; 42: 83.
31. Harris RJ, Pettitt AR, Schmutz C, *et al.* Granulocyte-macrophage colony-stimulating factor as an autocrine survival factor for mature normal and malignant B lymphocytes. *J Immunol* 2000; 164: 3887.
32. Iwasaki-Arai J, Iwasaki H, Miyamoto T, *et al.* Enforced granulocyte/macrophage colony-stimulating factor signals do not support lymphopoiesis, but instruct lymphoid to myelomonocytic lineage conversion. *J Exp Med* 2003; 197: 1311.
33. Stass S, Melvin MJ, Pui S, *et al.* Lineage switch in acute leukemia. *Blood* 1984; 64: 701.
34. Tsuruta N, Yatsunami J, Takayama K, *et al.* Granulocyte-macrophage-colony stimulating factor stimulates tumor invasiveness in squamous cell lung carcinoma. *Cancer* 1998; 82: 2173.
35. Yoon SO, Park SJ, Yoon SY, *et al.* Sustained production of H(2)O(2) activates pro-matrix metalloproteinase-2 through receptor tyrosine kinases/phosphatidylinositol 3-kinase/NF-kappa B pathway. *J Biol Chem* 2002; 277: 30271.
36. Bozinovski S, Jones JE, Vlahos R, *et al.* Granulocyte/macrophage-colony-stimulating factor (GM-CSF) regulates lung innate immunity to lipopolysaccharide through Akt/Erk activation of NFkappa B and AP-1 in vivo. *J Biol Chem* 2002; 277: 42808.
37. Chung TW, Lee YC, Kim CH. Hepatitis B viral HBx induces matrix metalloproteinase-9 gene expression through activation of ERK and PI-3K/AKT pathways: involvement of invasive potential. *FASEB J* 2004; 18: 1123.
38. Westermarck J, Kahari VM. Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J* 1999; 13: 781.
39. Ringshausen I, Dechow T, Schneller F. Constitutive activation of the MAPkinase p38 is critical for MMP-9 production and survival of B-CLL cells on bone marrow stromal cells. *Leukemia* 2004; 18: 1964.
40. Huang FM, Yang SF, Hsieh YS. Examination of the signal transduction pathways involved in matrix metalloproteinases-2 in human pulp cells. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2004; 97: 398.
41. Faehling M, Kroll J, Fohr KJ. Essential role of calcium in vascular endothelial growth factor A-induced signaling: mechanism of the antiangiogenic effect of carboxyamidotriazole. *FASEB J* 2002; 16: 1805.
42. McLaughlin AP, De Vries GW. Role of PLCgamma and Ca(2+) in VEGF- and FGF-induced choroidal endothelial cell proliferation. *Am J Physiol Cell Physiol* 2001; 281: C1448.
43. Mergler S, Dannowski H, Bednarz J, *et al.* Calcium influx induced by activation of receptor tyrosine kinases in SV40-transfected human corneal endothelial cells. *Exp Eye Res* 2003; 77: 485.
44. Merle PL, Usson Y, Robert-Nicoud M, *et al.* Basic FGF enhances calcium permeable channel openings in adult rat cardiac myocytes: implication in the bFGF-induced increase of free Ca2+ content. *J Mol Cell Cardiol* 1997; 29: 2687.
45. Raaijmakers HG, Van Den Bosch G, Boezeman J, *et al.* Single-cell image analysis to assess ABC-transporter-mediated efflux in highly purified hematopoietic progenitors. *Cytometry* 2002; 49: 135.
46. Peluso JJ. Basic fibroblast growth factor (bFGF) regulation of the plasma membrane calcium ATPase (PMCA) as part of an anti-apoptotic mechanism of action. *Biochem Pharmacol* 2003; 66: 1363.
47. Teh SH, Hill AK, Foley DA, *et al.* COX inhibitors modulate bFGF-induced cell survival in MCF-7 breast cancer cells. *J Cell Biochem* 2004; 91: 796.