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The granzyme B inhibitor PI-9 is differentially expressed in all main subtypes of pediatric acute lymphoblastic leukemias

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A B S T R A C T

Background and Objectives. The success of bone marrow transplantation in leukemia depends on graft-versus-leukemia (GvL) effects, mediated by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. These act by CD95L or granule cytotoxins, such as granzyme B (GrB) whose only known inhibitor is proteinase inhibitor 9 (PI-9). Since PI-9 protects cells from CTL, PI-9 may counteract GvL in leukemias. Our aim was to establish methods to analyze the expression and function of PI-9.

Design and Methods. We screened the most common pediatric ALL subsets, i.e. pre-B, common, and cortical T-ALL, for PI-9 expression, using a reverse transcription polymerase chain reaction (RT-PCR) approach which was correlated to semiquantitative and functional methods established in cell lines and patient probes.

Results. *In vitro*, PI-9 mediated resistance towards CTL and inhibited GrB-induced cleavage of caspase 3. In patient-derived ALL cells, PI-9 high and PI-9 low specimens were studied by flow cytometry, RT-PCR, *in vitro* GrB treatment and NK assay, demonstrating concordant results and PI-9-dependent target cell protection. Analysis of PI-9 in probes from ALL patients showed differential expression, but no correlation with immunotype.

Interpretation and Conclusions. Our data suggest that PI-9 in pediatric ALL is differentially expressed, without close correlation to subtype. Since PI-9 considerably alters GrzB and killer cell sensitivity, it may strongly influence the efficacy of GvL effects. The approaches applied here will allow evaluation of the expression and function of PI-9 in larger series of malignancies.

Key words: PI-9, cytotoxicity, leukemia, apoptosis, granzyme B.

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Following allogeneic bone marrow transplantation, graft-versus-leukemia (GvL) effects by cytotoxic T lymphocytes (CTL) and naturall killer (NK) cells are crucial for disease control.[1,2] The efficacy of the GvL effects depends on the sensitivity of the targets and on the killer/target ratio. In GvL, CTL and NK cells mainly act by granular enzymes, especially granzyme B and perforin.[1,2] Granzyme B is a serine protease that cleaves and thus activates multiple substrates in the target cells, including caspases 3 and 8, the anti-apoptotic regulator protein Bid, and the DNase complex ICAD, all leading to activation of the apoptosis cascade.[3-7]

PI-9, a 42 kDa human serpin that irreversibly inactivates granzyme B,[8-13] is the only known natural inhibitor of this protease. Cells that overexpress PI-9 acquire protection from granzyme B-dependent, but not CD95-dependent cytotoxicity.[9] PI-9 is expressed in the cytoplasm and nuclei of several cell types, including cytotoxic lymphocytes (T and NK cells), antigen-presenting cells (dendritic cells, macrophages, B cells), and cells at immunoprivileged sites.[13-18] Several leukemia cell lines used for tumor immunology studies, such as K562 and YT, are positive, while CEM, HL60 and U937 do not express PI-9.[9]

The present concept of the biological function of PI-9 is protection of cytotoxic or bystander cells from misdirected granzyme B at sites of inflammation.[9,13,14,18] However, several lines of evidence indicate that PI-9 expression might contribute to resistance of malignant cells towards killer cells, e.g. in the context of a GvL effect after bone marrow transplantation. Recently, it was demonstrated[19] that overexpression of SPI-6 — one of the murine

counterparts of human PI-9 — in lymphoma cells may lead to escape from CTL. Only descriptive studies have been performed in clinical specimens so far. In a series of Hodgkin's and non-Hodgkin's lymphomas, heterogeneous expression of the serpin was shown: levels were higher in T lymphomas than in B lymphomas, and there was some correlation between PI-9 expression and outcome in high-grade diseases.[20,21]

In this work, we first generated a neuroblastoma cell line, SH-EP, stably transfected with PI-9 or an empty vector, to establish methods for analyzing the expression and function of PI-9. The functional relevance of differential PI-9 expression was then analyzed in cell-free extracts from leukemia cell lines and primary patient derived leukemia cells.

Design and Methods

Cell lines and culture conditions

Cells were kept in RPMI 1640 medium (Life Technologies, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal calf serum (Conco, Wiesbaden, Germany), 12.5 mM HEPES (Biochrom, Berlin, Germany), 100 U/mL pernicillin/streptomycin solution (Life Technologies) and 2.0 mM L-glutamine solution (Biochrom). The cell extraction buffer (CEB) contained 50 mM PIPES (Sigma), pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT, Sigma), 10 μM cytochalasin B (Sigma) and 1 mM phenylmethylsulfonylfluoride (PMSF, Sigma).[22,23] The extract dilution buffer (EDB) contained 10 mM HEPES (Sigma), 50 mM NaCl, 5 mM EGTA, 2 mM $MgCl_2$ and 1 mM DTT.[22,23] The NK line YT and several other leukemia cell lines were purchased from the Deutsche Sammlung von Mikroorganismen und Zellinien (DMSZ, Braunschweig, Germany).[24] Fresh pre-treatment samples from patients with ALL were obtained after informed consent during the initial diagnostic procedures and frozen at -70°C, in 10% dimethylsulfoxide (DMSO, Sigma) and 90% fetal calf serum, or immediatedly lysed for RNA preparation as described below.

Cell-free extracts

The cell-free extracts were prepared basically following the protocol of Martin *et al.*[23] Cells (1×10^8) were washed in PBS twice, than once in CEB. The pellet was resuspended in 1 mL of CEB, then centrifuged at 4,000 rpm for 40s. The supernatant was discarded and 1 volume of pellet CEB was added. The cells were resuspended and kept on ice for 20 min to swell and then transferred to a 2 mL glass douncer (Wheaton, Millville, NJ, USA) and gently ruptured by about 20 strokes of the pestle. The efficacy of lysis was controlled by trypan blue staining. When most of the cells had been disrupted, the probe was diluted with 1 volume of EDB and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was taken and adjusted to a concentration of 10 or 20 mg/mL protein by dilution with EDB after determining the protein content in a photometer at 280 nm.

In vitro granzyme B assay of cell-free extracts and immunoblot

Twenty micrograms of extracts were incubated for 2h with granzyme B (Calbiochem, La Jolla, CA, USA) at the given concentrations at 37°C in 5% CO_2 and immediately frozen at -80°C. For immunoblots, probes were heated to 95°C for 3 min, than separated by 12% SDS-PAGE (Owl, Portsmouth, NH, USA) and electroblotted onto a nitrocellulose membrane (Amersham, Braunschweig, Germany). After blocking for 30 min in PBS supplemented with 2.5% (w/v) skimmed milk powder (Fluka Chemie, Buchs, Germany) and 0.1% Tween20 (Sigma), for immunodetection, the following antibodies were used: mouse anti-caspase 3 (Transduction Laboratories); monoclonal mouse anti PI-9, 7D8 (hybridoma supernatant), in a 1:10 ratio;[10] and anti α-tubulin (Oncogene). As secondary antibodies, horseradish-peroxidase-coupled goat anti-rabbit or anti-mouse (Santa Cruz Biotechnology) were used. An enhanced chemiluminescence system (Amersham) was used for detection.

Flow cytometry

For detection of intracellular PI-9, the PI-9-specific monoclonal mouse IgG1 antibody 2E7[10] was applied on cells fixed and permeabilized using the IntraPrep™ kit (Immunotech, Marseille, France), with FITC-labeled goat anti-mouse IgG (Serotec) as the secondary antibody. Analysis was done using a FACScan™ flow cytometer (Becton-Dickinson) according to the manufacturer's instructions. Mean fluorescence intensity (MFI) ratio was determined as the ratio between 2E7-stained cells and control-stained cells.

Cytotoxicity assay by two-color flow cytometry

Target cells were stained by 60 min incubation with calcein-AM (Molecular Probes, Eugene, OR, USA) — a green fluorescent intravital dye — at a concentration of 50 ng/mL and the washed with culture medium. Target cells (2×10^4) were mixed with effector cells in the given ratios in 96-well round-bottom microtiter plates (Falcon, Becton Dickinson, NJ, USA) in a volume of 100 μL, centrifuged for 5 minutes at 1000 rpm and incubated for 4h at 37°C in 5% CO_2. Cells were then stained with propidium iodide (PI, Sigma;

3 μL per pellet of a 20 μg/mL solution of PI in NaCl 0.9%) and cytotoxicity was assessed in a two-color flow cytometry assay as PI positivity of calcein-AM positive targets using a FACScan flow cytometer (Becton-Dickinson). Per cent specific apoptosis was calculated as follows:

$$\% \text{ specific apoptosis} = \frac{\text{PI-positivity (killing assay) minus spontaneous PI-positivity}}{\text{100 minus spontaneous PI-positivity}} \times 100\%$$

Reverse transcription polymerase chain reaction

For isolation of RNA and RT-PCR, the Qiagen (Hilden, Germany) and RNA-PCR Perkin-Elmer (Foster City, CA, USA) kits were used, according to the manufacturers' instructions. The following primer pairs were used:[12] β-actin 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA and 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG; PI-9:5'-TCT GCC CTG GCC ATG GTT CTC CTA and 5'-CTG GCC TTT GCT CCT CCT GGT TTA, amplifying a 471 bp fragment of human PI-9. Primers were synthesized by Interactiva, Ulm, Germany. Amplification was done in 32 cycles with an annealing temperature of 58°C.[11] Electrophoresis was done using 100 Volt and 400 mA for 40 min on a 0.5% agarose gel; for detection, we used SybrGreen™ (FMC Bioproducts, Rockland, ME, USA) and an ultraviolet camera.

Vector construction and transfection

The vector construct containing PI-9 was prepared as follows. Total RNA from peripheral blood mononuclear cells from healthy donors was subjected to RT-PCR using the primers[11] 5'(5'-GTGGCAGGCCCTGCATCA-3'), linked to the Eco1 recognition sequence GAATTC, and 3'(5'-CACCCTTTATGGCGATGA-3'), linked to the BamH recognition sequence GGATCC (Perkin-Elmer kit, pfu-Turbo Stratagene, La Jolla, CA, USA), resulting in a PCR product (containing nucleotides 87-1240 of PI-9 cDNA, OMIM entry No. *601799) which contains the complete coding region of the protein. The PCR product was then cloned into the vector pIRESneo2 (Clontech) and amplified by XL10-Gold™ Ultracompetent Cells (Stratagene). Identity was confirmed by sequencing (TopLab, Martinsried, Germany) which revealed exactly the same sequence as published (OMIM). The vector was then amplified by XL10-Gold™ Ultracompetent Cells, extracted by the Qiagen Plasmid Maxi Kit (Qiagen) and transfected into SH-EP cells, using the Gene Porter system (GTS, San Diego, CA, USA). As controls, cells were transfected with empty vector. The pIRESneo2 vector contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus which permits translation of two open reading frames from one messenger RNA leading to continuous expression of the neomycin resistance gene together with the gene of interest. After 48h, neomycin (Sigma) was added at 600 μg/mL for one week, then 400 μg/mL. To enhance expression of the gene of interest, the neomycin concentration was later increased to 2400 μg/mL which was tolerated well by the cells.

Results

PI-9 confers stably transfected SH-EP cells resistance to killing by cytotoxic cells

To demonstrate the function of PI-9 *in vitro*, the PI-9 negative neuroblastoma cell line SH-EP was stably transfected with a vector containing PI-9 (SH-EP-pIRES-PI-9), or the control vector (SH-EP-pIRES). High expression of PI-9 was shown in the SH-EP-pIRES-PI-9 cells by intracellular flow cytometry and RT-PCR (Figures 1A,B). PI-9-transfected cells were resistant to killer cells as demonstrated by a two-color flow cytometric cytotoxicity assay, using YT cells as effectors. Cytotoxic activity of YT cells towards SH-EP cells was completely inhibited by EGTA, indicating that it depends on the granular pathway (Figure 1C). In the PI-9-transfected SH-EP cell line, we found significant resistance towards YT-mediated killing in 1-hour and a 4-hour assays (Figures 1C,D). Similar results were seen using short-term activated NK cells as effectors. Death induced by doxorubicin and serum starvation was unaffected (*data not shown*).

PI-9 in transfected neuroblastoma cells and in patient-derived leukemia cells inhibits granzyme B-induced cleavage in vitro

In parallel with the transfected neuroblastoma cells, we used flow cytometry to study expression of PI-9 in two fresh leukemia cell samples derived from patients (patients 1 and 2). Cells from patient 1 had much higher PI-9 expression than those from patient 2, as evaluated by protein levels (Figure 2A). This was confirmed by RT-PCR (*data not shown*). PI-9 function was then demonstrated *in vitro*, using cell-free extracts incubated for 2h at 37°C in the presence of different doses of granzyme B (25 μg/20 μL extracts and 0, 0.2, 0.5 and 1.0 μg/mL granzyme B), followed by immunoblotting after SDS-PAGE. Cell-free extracts from SH-EP-pIRES, SH-EP-pIRES-PI-9 and the two pediatric leukemia patients were studied on the same blot with

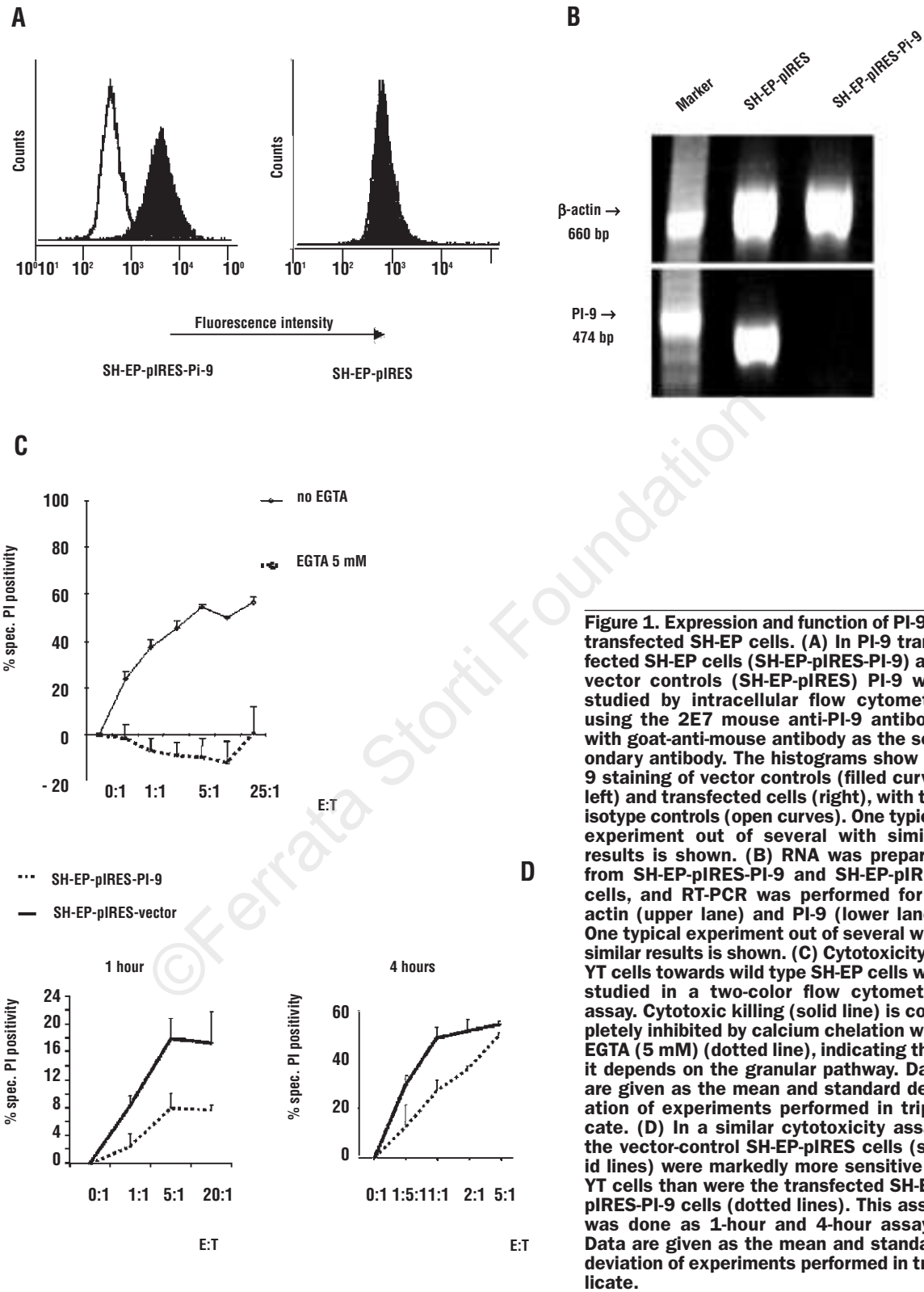

Figure 1. Expression and function of PI-9 in transfected SH-EP cells. (A) In PI-9 transfected SH-EP cells (SH-EP-pIRES-PI-9) and vector controls (SH-EP-pIRES) PI-9 was studied by intracellular flow cytometry using the 2E7 mouse anti-PI-9 antibody with goat-anti-mouse antibody as the secondary antibody. The histograms show PI-9 staining of vector controls (filled curve, left) and transfected cells (right), with the isotype controls (open curves). One typical experiment out of several with similar results is shown. (B) RNA was prepared from SH-EP-pIRES-PI-9 and SH-EP-pIRES cells, and RT-PCR was performed for β-actin (upper lane) and PI-9 (lower lane). One typical experiment out of several with similar results is shown. (C) Cytotoxicity of YT cells towards wild type SH-EP cells was studied in a two-color flow cytometric assay. Cytotoxic killing (solid line) is completely inhibited by calcium chelation with EGTA (5 mM) (dotted line), indicating that it depends on the granular pathway. Data are given as the mean and standard deviation of experiments performed in triplicate. (D) In a similar cytotoxicity assay, the vector-control SH-EP-pIRES cells (solid lines) were markedly more sensitive to YT cells than were the transfected SH-EP-pIRES-PI-9 cells (dotted lines). This assay was done as 1-hour and 4-hour assays. Data are given as the mean and standard deviation of experiments performed in triplicate.

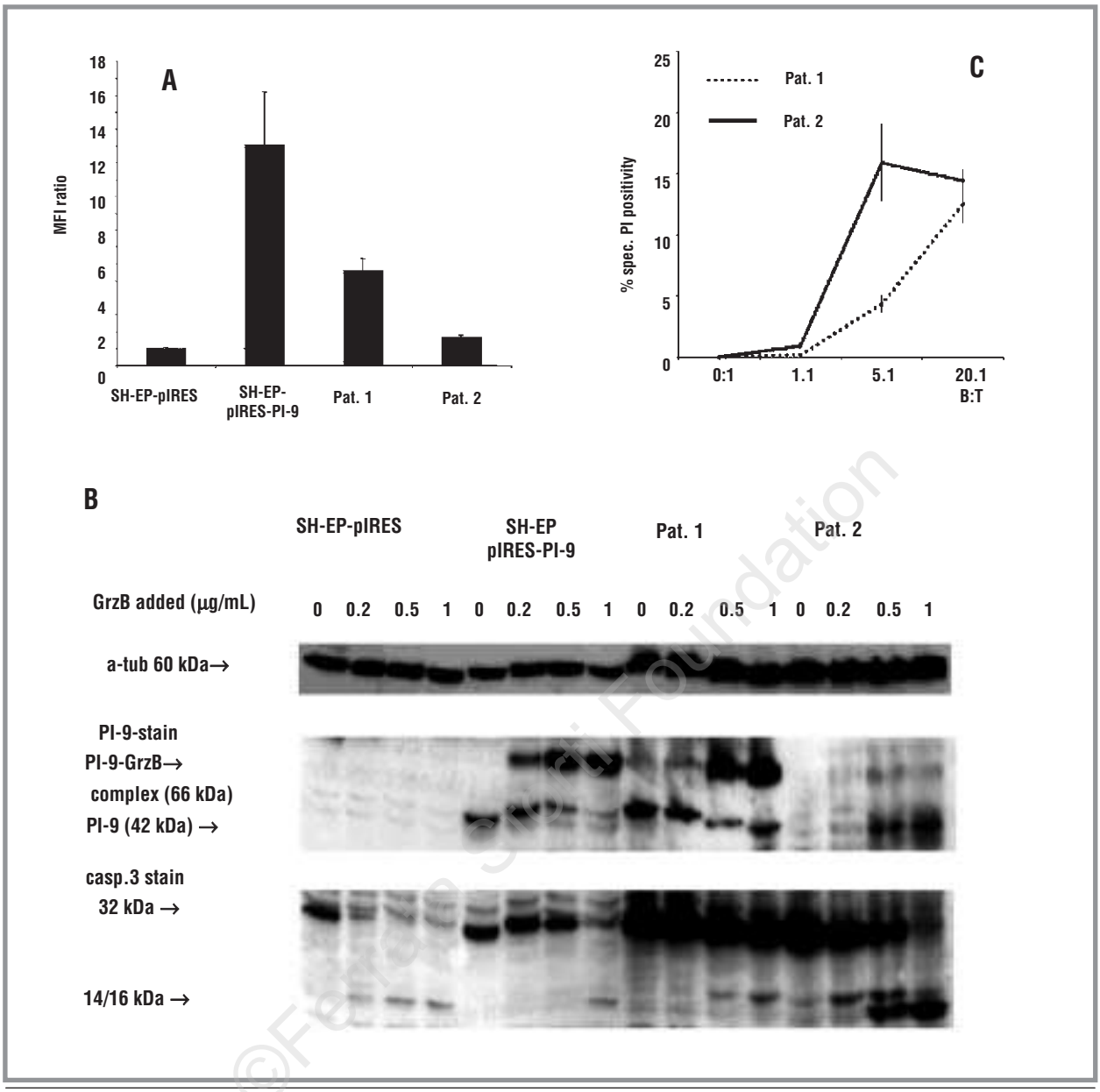

Figure 2. Expression and function of PI-9 in selected patient-derived leukemia cells, in comparison with transfected SH-EP cells. (A) SH-EP-pIRES-PI-9 and SH-EP-pIRES cells were analyzed by flow cytometry in parallel with fresh leukemia cells from two patients (patient 1 and patient 2), and the mean fluorescence intensity ratio was calculated. Data are given as the mean and standard deviation of experiments carried out in triplicate. (B) Cell-free extracts of SH-EP-pIRES-PI-9 and SH-EP-pIRES cells and leukemia cells from the two patients were prepared as described in the Design and Methods section.The extracts were then incubated *in vitro* for 2 h at 37°C, with increasing doses of granzyme B (25 μg/20μL extracts, 0, 0.2, 0.5, 1.0 μg/mL granzyme B). After SDS-PAGE, immunoblotting was performed showing α-tubulin (60 kDa) as the control, then PI-9 (42 kDa) and granzyme B/PI-9 complexes (60-72 kDa), stained by the monoclonal mouse anti-PI-9 antibody 7D8, with no signal from the vector controls. In parallel, caspase 3 (32 kDa proform, 14/16 kDa active fragment) was stained. As the doses of granzyme B increased, the PI9/GrB complex (66 kDa) appeared in the PI-9 positive cell extracts, and caspase 3 was cleaved to its active fragments. Caspase cleavage required higher doses of granzyme B in PI-9 positive extracts than in PI-9 negative ones. One typical experiment out of several with similar results is shown. (c) Cells from two patients with ALL were subjected to cytotoxic killing using the NK cell line YT – which is known to kill targets via granzyme B – as effectors in a co-incubation assay. Cytotoxic killing was assessed using the flow cytometry assay. Cells from patient #1, displaying a higher content of PI-9, were more resistant towards YT cells than those of patient #2. The mean and range of duplicate experiments are shown.

α-tubulin stained as the control (60 kDa).

Using the monoclonal mouse anti-PI-9 antibody 7D8, expression of the 42 kDa native serpin could be demonstrated in SH-EP-pIRES-PI-9 cells, and formation of the PI-9/granzyme B complex at 65-70 kDa, appearing upon incubation in the presence of granzyme B, was

visible. These signals were absent for the vector controls (Figure 2B). As a classical target molecule of granzyme B, we studied caspase 3 on the same blots, using an antibody that stains both the uncleaved proform (32 kDa) and the two main cleavage fragments (14/16 kDa). Much higher concentrations of granzyme B were required to achieve caspase 3 cleavage in SH-EP-pIRES-PI-9 than in the controls (Figure 2B). We found a similar pattern in the two patient probes (one PI-9 high, the other PI-9 low): in the PI-9 high cells (patient #1), the uncleaved caspase 3 proform persisted even in the presence of high granzyme B concentrations (1 μg/mL), while in the PI-9 low extracts (patient #2), complete caspase cleavage was already achieved at low levels of granzyme B (0.5 μg/mL). Roughly, the amount of granzyme B needed to cleave caspase 3 was at least five times the amount needed in PI-9 low cells. These data show that in primary leukemia cells, PI-9 can inactivate granzyme B, and suggests that high PI-9 levels may correlate with resistance to killing by cytotoxic lymphocytes.

Correlation between PI-9 expression and sensitivity towards YT-cell killing in patient-derived leukemia cells

We then analyzed the sensitivity of the two patient-derived leukemia cell samples towards cytotoxic killing using the flow cytometric killing assay. The target cells, cryopreserved cells from the two patients, and the effector cells, the NK cell line YT, were incubated together for 4 h. Cells from patient #1, with a higher content of PI-9, were more resistant to YT cells than were the cells from patient #2 (Figure 2C), especially at the effector/target ratio of 5:1.

PI-9 expression in a series of leukemia cell lines (flow cytometry and RT-PCR)

To correlate the semiquantitative methods of flow cytometry and RT-PCR with each other and with published data, a series of leukemia cell lines was studied using both methods in parallel. We found a good correlation between flow cytometry and RT-PCR for semiquantitative detection of PI-9, showing high PI-9 expression in YT and moderate expression in Bjab, HL-60, Raji, and K562 cells. RT-PCR gave a stronger signal at low levels, and flow cytometry a better differentiation at high levels of expression (Figures 3 A,B).

PI-9 expression in a series of pediatric ALL (RT-PCR)

The RT-PCR approach was used to semiquantitatively measure PI-9 expression in 19 samples of different *de novo* pediatric acute lymphoblastic leukemias (4 pre-B-ALL, 4 cortical T-ALL, 1 mature T-ALL, 10 common-ALL) (Figure 3C). Including SH-EP-pIRES, SH-EP-pIRES-PI-9 cells as controls, we found a wide range of levels of PI-9 expression, and in each of the immunological subtypes (except mature T-ALL) both high and low PI-9 expressing cases were identified. Samples from 2 patients with cortical T-ALL who later experienced early relapse were included: one of these was a prednisone poor responder and had high PI-9 expression, the other relapsing patient had low PI-9 expression.

Discussion

PI-9 expression and function have been analyzed in a number of model systems,[9,11,18,19] but the relevance of this finding in the clinical setting of leukemia is still unclear. Here — using functional assays established in a transfected neuroblastoma cell model — we demonstrate that differences in PI-9 expression in patient-derived pediatric leukemia samples are sufficient to shift the sensitivity towards granzyme B-mediated caspase activation. The amount of granzyme B needed to cleave caspase 3 in leukemia samples with high PI-9 expression was about five times the amount needed in PI-9 low cells. This clearly suggests that increased PI-9 expression has an impact on CTL resistance in GVL, although it is well-known that the sensitivity of target cells to killer cells also depends very strongly on other factors, mainly the killer/target recognition mechanisms which are mediated by a number of surface antigens.

Recent data from lymphoma studies indicated that levels of PI-9 expression might characterize T or B subtypes. However, we found no clear correlation with immunophenotype in pediatric ALL. In the lymphoma studies mentioned above,[20] high and low PI-9 expression were found in nearly all variants of the disease. High PI-9 expression tended to correlate with a worse prognosis. In a recent study focusing on large cell anaplastic lymphoma,[21] a correlation was described between the more malignant anaplastic large cell kinase (ALK) negative subtypes and high PI-9 expression. PI-9 positive and negative subtypes were found in series of both melanoma and carcinoma cell lines,[19] indicating that heterogeneity of PI-9 expression is not restricted to diseases of the hematopoietic system. Our series, containing only one prednisone-poor responder and two patients who relapsed early, does not allow conclusions to be drawn on the prognostic relevance of PI-9 expression in primary disease.

Physiologically, the regulation of PI-9 expression is mediated mainly by nuclear factor-κ B and activator protein 1,[25] and is thus influenced by the stimulation status of the cell and cytokine concentrations. However, physiological concentrations of cytokines lead to

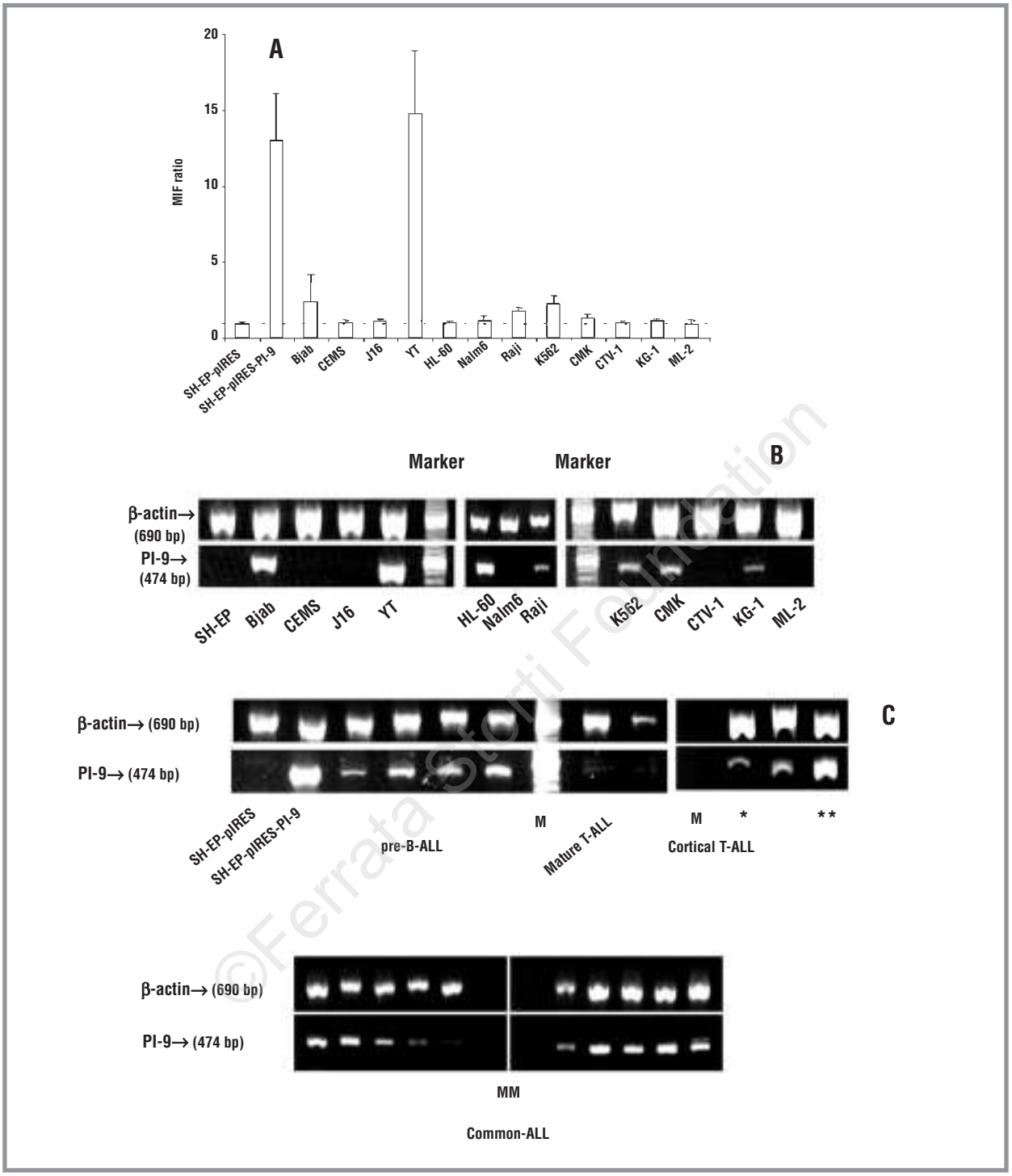

Figure 3. PI-9 expression of cell lines and patient-derived leukemia cells, analyzed by flow cytometry and RT-PCR (A) PI-9 expression was analyzed by flow cytometry in a panel of cell lines, including the above mentioned transfected neuroblastoma cell lines SH-EP-pIRES-PI-9 and SH-EP-pIRES, and the mean fluorescence intensity ratio was calculated. Data are given as the mean and standard deviation of experiments carried out in triplicate. (B) RNA was prepared from the same panel of cell lines as shown in Figure 3A (using wild-type SH-EP cells), and RT-PCR was performed for β-actin (upper lane) and PI-9 (lower lane). One out of several experiments with similar results is shown. The correlation between flow cytometry and RT-PCR results was good. (C) RNA was prepared from fresh lymphoblastic leukemia cells from 19 pediatric patients (4 pre-B-ALL, 4 cortical T-ALL, 1 mature T-ALL, and 10 common-ALL), and RT-PCR was performed for β-actin (upper lane) and PI-9 (lower lane), with SH-EP-pIRES-PI-9 and SH-EP-pIRES as controls. The patient marked with * later experienced early relapse of leukemia. The patient marked by **had a prednisone-poor response and early relapse of disease. One out of several experiments with similar results is shown. The PCR markers are labeled by M.

rather slow and moderate changes of PI-9 in lymphocytes, as compared to other activation-related molecules.[13] Therefore, although PI-9 expression in leukemia cells might be influenced by external factors to some degree, it mainly represents an intrinsic property of the cells. PI-9 is relatively equally expressed in all subsets of normal lymphocytes.[13] Therefore, our observation that expression of PI-9 is very heterogeneous in their malignant counterparts, is noteworthy.

In summary, our data suggest that PI-9 in pediatric ALL is differentially expressed, without close correlation to subtype. Although it seems not to be a prognostic factor in general, it may strongly influence the efficacy of the GvL effect, since it alters sensitivity to granzyme B and killer cells quite considerably. The approaches applied here will allow the expression and function of PI-9 to be evaluated in a larger series of malignancies.

CFC: basic conception and design of the study, performed the experiments (work on the PI-9-containing vector was performed together with AU), drafted the article, final approval; AU: designed and produced the PI-9-containing vector and performed all experiments using the vector, critical revision of the paper, final approval; PB: conception of the study, production of the anti-PI 9-antibodies, analysis and interpretation of data, critical revision with substantial contribution to its content, repeated critical revision of the paper, final approval; KMD: basic conception of the study, critical revision with contribution to its content, final approval.

The authors reported no potential conflicts of interest.

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