Protein tyrosine phosphatases regulate physiological processes including growth, differentiation, metabolism and the cell cycle. Together with tyrosine kinases, they control the phosphorylation state of tyrosine residues of signaling proteins. An increased level of protein phosphorylation results in abnormal proliferation and many cancer types show a mutation or deletion of a protein tyrosine phosphatase gene. In this study we evaluated the protein tyrosine phosphatase activity in acute leukemia patients. Tyrosine phosphatase activity in bone marrow mononuclear cells of acute leukemia patients was measured using a tyrosine phosphatase assay system kit and compared with a control group. We found that tyrosine phosphatase activity in acute leukemia patients was high compared to the controls. According to subgroups of acute leukemia, tyrosine phosphatase activity in the AML-M2 subgroup was high compared to the controls. The effect of increased level of protein tyrosine phosphatase activity on leukemogenesis needs further evaluation. Studies in a large group of patients are needed to emphasize the importance of tyrosine phosphatase activity in acute leukemia patients.

Key words: tyrosine phosphatase activity, acute leukemia.

Evaluation of protein tyrosine phosphatase activity in patients with acute leukemia

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Introduction

Protein and membrane lipid phosphorylation play a key role in the intracellular signal transduction system [1]. The phosphorylation activity is regulated by the interaction of protein kinase and protein phosphatase [2]. Protein phosphatases are divided into two main groups [2].
1. Phosphotyrosine-containing peptide or enzymes that remove phosphate from proteins (protein tyrosine phosphatase),
2. Phosphoserine- or phosphothreonine-containing peptide or enzymes that remove phosphate from proteins (protein serine/threonine (Ser/Thr) phosphatase).

Protein tyrosine phosphatases have important physiologic cellular functions due to their positive and negative effects on signal transduction pathways. As a result, any disruption of protein tyrosine phosphatase activity contributes to the pathogenesis of many diseases including cancer [2]. A study evaluating protein Ser/Thr phosphatase expression in human leukemic cells has been published [3]. On the other hand, a study has been conducted on promyelocytic leukemia cell lines, with regards to protein tyrosine phosphatases [4], whereas no study evaluating the activity of protein tyrosine phosphatase in human leukemic cells has been reported in the literature.

Our aim in this study was to evaluate protein tyrosine phosphatase activity in mononuclear cells isolated from patients diagnosed with acute leukemia. Evaluation of the other side of the phosphorylation equilibrium in selected patients may improve current understanding of leukemogenesis and provide a new perspective on treatment approaches.

Material and methods

Material

RPMI (e.g. Sigma Cat. No: 6504), physiologic saline, Ficoll-Paque (density 1.077 g/ml) (e.g. Stem Cell Technologies Cat. No: 07907), sterile pipettes, 15–50 ml conical tubes, Tyrosine Phosphatase Assay System (Promega V2471), homogenization buffer (24 mM tris-HCL: pH: 7.5, 10 mM β-mercaptoethanol, 2 mM EDTA, 1 mM benzamidine, 0.1 mM phenyl-methylsulfonyl fluoride, $20\,\mu$g/ml leupeptin, $1\,\mu$M pepstatin A, $1\,\mu$g/ml aprotinin) [5], AK Sephadex G-25 chemical material required for the storage buffer (10 mM Tris, pH: 7.5, 1 mM EDTA, 0.02% sodium azide), material required for the reaction buffer (25 mM imidazole, 2 mM EDTA, 50 mM NaCl, 5 mM DTT) [6].

Methods

A total of 36 patients who visited the hematology clinic and the emergency service within a three-month period and whose clinical and laboratory data and the peripheral smear examination results pointed to a diagnosis of acute

leukemia were enrolled in this prospective study. The definitive diagnosis of these patients was made by aspiration of the required bone marrow from the patients and the addition to this sample of another aspiration sample of 10 cc of bone marrow with heparin after obtaining informed consent from the patients. All patients' blast counts were evaluated. After making a definitive diagnosis of acute myeloid leukemia (AML) from the bone marrow examination, AML subtypes were determined. The mononuclear cells were separated from these samples using the Ficoll-Hypaque density gradient centrifugation method and stored at –80°C until the study day. As a control group, a sample of 10 cc of bone marrow with heparin was collected, after obtaining informed consent from 10 healthy and suitable individuals who agreed to be volunteers as donors for allogeneic stem cell transplantation. Mononuclear cells were separated from these samples using Ficoll-Hypaque density gradient centrifugation and stored at –80°C until the study day.

Mononuclear cell isolation

The heparinized bone marrow sample which was aspirated from the posterior iliac bone of the patients and volunteers (50 U/ml heparin) was diluted at a ratio of 1 : 2 in sterile physiologic saline. Ficoll-Paque was put into 15 ml conical tubes, 1 : 3 of the blood volume. The diluted bone marrow aspirate was then slowly added to the medium. Ficoll and bone marrow containing tubes were placed at room temperature and centrifuged at 400 g for 30 minutes. After the centrifugation procedure plasma was found to be raised at the top, mononuclear cells settled in the middle, Ficoll below, while erythrocytes and granulocytes settled at the very bottom. Mononuclear cells were then collected while the other parts were thrown away. Ficoll, known to be toxic to cells, was warded off after centrifuging the collected cells at least twice with serum free culture medium five times the volume. A mononuclear cell suspension of bone marrow origin was thus obtained following these procedures.

Phosphatase activity measurement

The Promega non-radioactive Tyrosine Phosphatase Assay System kit was used for phosphatase activity measurement. The method is based on measuring the absorbent change at a suitable wavelength, generated after formation of a reaction mixture of molybdate : malachite green-phosphate complex of the free phosphate.

The synthetic peptides END(pY)INASL (phosphopeptide-1) [7] and DADE(pY)LIPQQG (phosphopeptide-2) [8], supplied as substrates in the measuring kit, were used to measure the enzyme activities of tyrosine phosphatases.

Phosphopeptide-1, which was supplied in the kit, was regulated to 1 mM using 895 μl of Phosphate-Free Water, whereas phosphopeptide-2 was regulated to 1 mM using 735 μl of Phosphate-Free Water. 5 μl of this regulated substrate was sufficient for general usage.

The 50 μl Molybdate Dye+ Molybdate Dye Additive mixture required for every 50 μl reaction was prepared. This mixture was freshly prepared on the day of the experiment. 10 μl of Molybdate Dye Additive was added to every 1 ml of Molybdate Dye Solution.

The 1 mM Phosphate Standard was diluted with the supplied Phosphate-Free Water, in order to prepare phosphate stock standard. This standard was diluted 1 : 20 to generate a solution containing 50 pmol phosphate per microliter.
Test procedures:

1. Mononuclear cells of patients and the controls stored at –80°C were removed and unfrozen.
2. Phosphatase storage buffer (24 mM tris-HCL: pH: 7.5, 10 mM -mercaptoethanol, 2 mM EDTA, 1 mM benzamidine, 0.1 mM PMSF, 20 μg/ml leupeptin, 1 μM pepstatin A, 1 μg/ml aprotinin) up to 3 mL was added to 1 g of tissue of these cells and homogenized at +4°C for 30 s using a homogenization device.
3. The homogenized lysate was centrifuged at 100 000× g at 4°C for 1 hour to remove particulate matter. The cytosolic fraction was obtained by removing the supernatant portion.
4. 10 ml of deionized water was added to the Spin Column and allowed to drain. 10 ml of resuspended Sephadex® G-25 slurry was pipetted into the Spin Column and it was allowed to drain by gravity into a spare 50 ml tube.
5. 10 ml of phosphatase storage buffer was added to the column.
6. The column was allowed to drain by gravity, the flow-through liquid was removed from the tube, and then centrifuged at 600× g for 5 min at 4°C using a spare 50 ml tube to remove the remaining buffer surrounding the Sephadex® beads.
7. 250 μl of cell lysate was added to the column.
8. The column was centrifuged at 600× g for 5 min at 4°C. The sample lysate in the storage buffer was left at the bottom of the reservoir in the original volume. 60 μl of cell lysate was separated for total protein analysis.
9. Appropriate phosphate standards were made by diluting the 1 mM Phosphate Standard with the supplied Phosphate-Free Water. The standard was diluted 1 : 20 to generate a solution containing 50 pmol phosphate per μl (50 μM). Wells were prepared containing 0, 100, 200, 500, 1000 and 2000 pmol free phosphate and 1X reaction buffer in 50 μl for use as a standard curve.
10. 10 μl of experimental buffer (25 mM imidazole, 2 mM EDTA, 50 mM NaCl, 5 mM DTT) was mixed in 5 μl of 1 mM phospholipid wells (preventing the formation of air bubbles) and incubated at 37°C for 3 min.
11. The reaction was started by adding 35 μl of enzyme sample to the wells and incubating at 37°C for 3 min.
12. The reaction was stopped by adding 50 μl Molybdate Dye/Additive mixture to the wells.
13. The 96-well plate was placed at room temperature and incubated for 15 min until color development.
14. The optical density of the samples was read using a plate reader with a 630 nm filter.
15. Phosphatase activity was calculated as protein by comparing with the phosphate standard curve (for example, total protein should previously be measured in cell lysates).

As a summary, tyrosine phosphatase enzyme in the samples reacts with tyrosine containing synthetic peptides, and removes phosphate groups from the peptides. The developing phosphate turns to a green color with the molybdate dye/additive mixture, and the resulting absorbent change is

measured at an appropriate wavelength. Tyrosine phosphatase activities are then evaluated using the previously prepared phosphate standard curve.

Total protein measurement in cell lysates

The Biured Protein assay was used for the measurement of total protein concentration in cell lysates.

Statistical analysis

The Kolmogorov-Smirnov test was used to test for a normal distribution of the group with regards to protein tyrosine phosphatase activity. The Student t-test was used for the comparison of two groups for data conforming to a normal distribution, whereas the Mann-Whitney U test was used for those not conforming to a normal distribution. Data obtained from the measurement were expressed as arithmetic mean ±standard deviation. $p < 0.05$ was considered as statistically significant.

Results

Patients' blast counts were evaluated (Fig. 1).

Numbers of patients in different AML subtypes were as follows: 21 in AML-M2, 5 in AML-M3, 5 in AML-M4, 2 in AML-M5, 2 in biphenotypic leukemia.
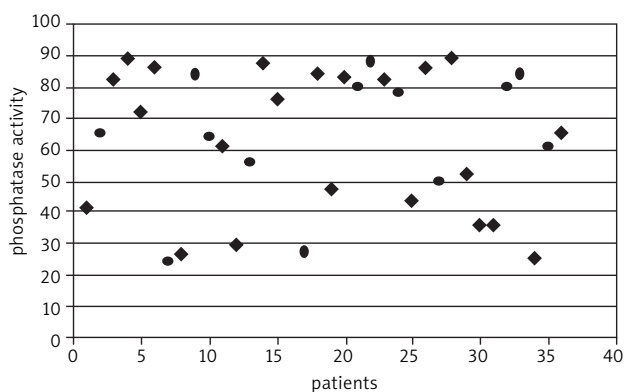

Fig. 1. Blast counts of patients

Phosphopeptide-1 (END(pY)INASL) and phosphopeptide 2 (DADE(pY)LIPQQG) of the patients and controls were calculated using the tyrosine phosphatase activity values pmol/min/μg protein. The tyrosine phosphatase activity mean of all patients for phosphopeptide-1 and phosphopeptide 2 were compared with those of the controls. Comparison of the mean of all patients with that of the controls for phosphopeptide-1 was found to be statistically significant, whereas no statistical significance was found when the evaluation was made for phosphopeptide-2 (Fig. 2).

Comparison of AML subtypes of the patients with the control group in respect of tyrosine phosphatase activities for phosphopeptide-1 demonstrated that there was a statistically significant difference between the AML-M2 subgroup and the controls. However, no difference was found amongst the other AML subtypes (Fig. 3). Similarly, results demonstrated a statistically significant difference between the AML-M2 subgroup and the controls in respect of tyrosine phosphatase activities for phosphopeptide-2, whereas no difference was reported amongst the other AML subtypes (Fig. 4).
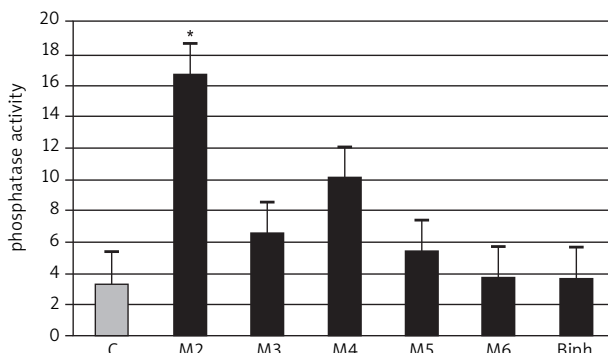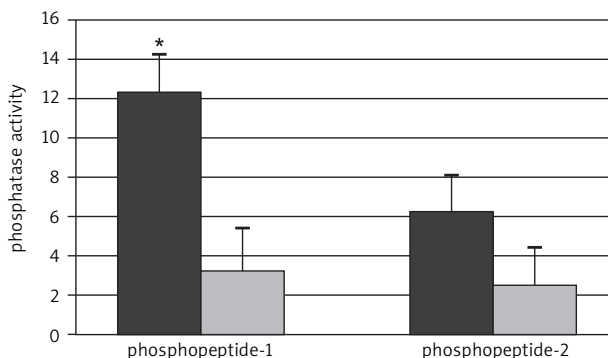

* Comparison of AML-M2-control group with tyrosine phosphatase activity mean.
* $p < 0.05$ black column patients, grey column controls; C: Controls, M2: AML M2 M3: AML M3, M4: AML M4, M5: AML M5, M6: AML M6, Biph: Biphenotypic leukemia.

Fig. 2. Comparison of AML subtypes with the control group in respect of tyrosine phospata se activities for phosphopeptide-1
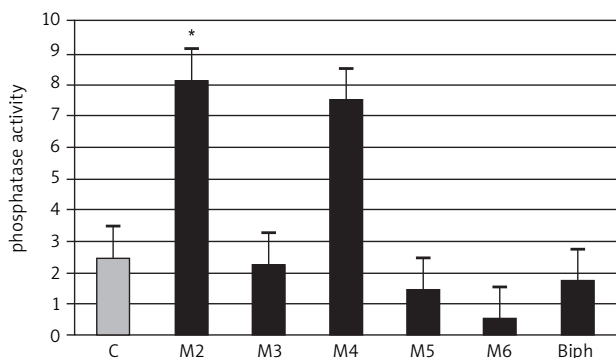
* Comparison of AML-M2-control group with tyrosine phosphatase activity mean.
* $p < 0.05$ black column patients, grey column controls; C: Controls, M2: AML M2, M3: AML M3, M4: AML M4, M5: AML M5, M6: AML M6, Biph: Biphenotypic leukemia.

Fig. 3. Comparison of AML subtypes with the control group in respect of tyrosine phospata se activities for phosphopeptide-2

* Comparison of the tyrosine phosphatase activity mean of all patients AML subtypes with that of the control group for phosphopeptide-1.
* $p < 0.05$ black column patients, grey column control.

Fig. 4. Comparison of the tyrosine phosphatase activity mean of all patients AML subtypes with that of the control group for phosphopeptide-1 and phosphopeptide-2

Discussion

Protein tyrosine phosphorylation and dephosphorylation play important roles in the regulation of many cellular processes including cell proliferation, differentiation and intercellular communication. Protein tyrosine kinases and protein tyrosine phosphatases are enzymes responsible for the regulation of protein tyrosine phosphorylation. About 500 protein tyrosine phosphatases are said to have been coded in the human genome [9]. Protein tyrosine phosphatases constitute a large enzyme family with varied substrate specificities, arrangements and expression [10].

Protein tyrosine phosphatases have both suppressing and stimulating effects in cancer-related signal transduction. Abnormality in protein tyrosine phosphatase activity has been demonstrated in some cancer types associated with tumor growth [11]. The oncogenic activity of protein tyrosine phosphatases has best been shown in hereditary and sporadic leukemia and to a lesser extent in the mutational activation of SHP2, which leads to the development of solid tumors [11].

In this study, we used two synthetic phosphoprotein substrates (DADE(pY)LIPQQG and END(pY)INASL) to measure protein tyrosine phosphatase activity of mononuclear cells obtained from the bone marrow samples of patients with acute leukemia. Experimentally synthesized substrates are used for the evaluation of protein tyrosine phosphatase activity due to absence of stoichiometrically phosphorylated substrates [8]. DADE(pY)LIPQQG and END(pY)INASL were chosen due to their kinetic advantages and also because they possess various features of a general substrate for various protein tyrosine phosphatases [7, 8].

All protein tyrosine phosphatases contain at least one catalytic domain which is made up of approximately 240 amino acids present in the active region known as the C(X)5R signature motif [9]. Substrate specificity of the phosphatase family provides a great opportunity for the dephosphorylation of phosphoproteins in the catalytic domain by variation [12]. The most important factor determining substrate specificity in the catalytic domain is amino acid arrangement [8]. Although protein phosphatases have different substrate specificities, they all use the same catalytic mechanism when removing phosphate groups from peptides.

In this study, which to the best of our knowledge is the first in which tyrosine phosphatase activity has been evaluated in acute leukemia patients, tyrosine phosphatase activity of leukemia patients was found to be increased when compared to the control group, in respect of phosphopeptide-1 and phosphopeptide-2. However, this increase was found to be statistically significant for phosphopeptide-1, whereas it was not significant for phosphopeptide-2. Although protein tyrosine phosphatases use the same catalytic mechanism, the difference in results obtained for the two phosphopeptides may be attributed to the difference in substrate specificity of the catalytic domain of the phosphatases. On the other hand, the phosphotyrosine position in the peptide is also said to affect protein tyrosine phosphatase activity [8]. This condition is also reported to be another reason for different results obtained for the two phosphopeptides with different structures.

The phosphorylation of critical substrates is responsible for regulating the direction of leukemic cell proliferation or differentiation pathways in the cell cycle [12]. In the study by Ayd n et al., where the effect of methylprednisolone on leukemic differentiation was reported to be due to the upregulation of PP2A, hypophosphorylation was reported to inhibit proliferation and to induce differentiation [13].

In the study conducted by Kraft et al., where the activity of tyrosine kinase and phosphotyrosine phosphatase in the human promyelocytic leukemia HL-60 cell lines and polymorphonuclear cells was evaluated, the activity of tyrosine kinase and phosphotyrosine phosphatase in polymorphonuclear cells was found to be higher compared to HL-60 cell lines. In the said study, this enzymatic activity was reported to be increased in myeloid differentiation [4]. The study is different from our study in the sense that tyrosine phosphatase activity was investigated only in promyelocytic leukemia and compared with polymorphonuclear leukocytes.

In another study, by Xu et al., Shp2 [Src homology 2 (SH2) domain-containing phosphotyrosine phosphatase 2] expression was investigated in 20 patients with AML, 12 patients with acute lymphocytic leukemia (ALL), 18 with chronic myeloid leukemia (CML), one with chronic lymphocytic leukemia (CLL), and one patient diagnosed with biphenotypic leukemia [14]. Comparison of primary leukemia cells with normal hematopoietic progenitor cells demonstrates that Shp2 expression significantly increased in primary leukemia cells. The increased tyrosine phosphatase activity in patients with leukemia in our study also suggests that increased tyrosine phosphatase activity may contribute to leukemogenesis.

In our study, the tyrosine phosphatase activity of the AML-M2 subgroup was found to be higher for both phosphopeptides in respect of AML subtype, compared to the control group. However, no statistically significant difference was observed with the other AML subtypes. This was attributed to the fact that most patients enrolled in the study were of AML-M2 subtype, and the number of patients in the other groups must have been small. This condition suggests that similar results may be obtained with the other AML subgroups when further studies are performed involving a larger patient population.

In the study by Yamamoto et al., where four different serine/tyrosine phosphatase PP1, PP2A, PP2B, PP2C expressions were evaluated in human leukemic cells, it was demonstrated that every phosphatase was expressed at different rates in different leukemia cell types. Although the reason for this difference was not well understood, regulation was suggested to be due to the level of RNA transcription, and not due to mRNA stabilization. This difference is said to play an important role in the regulation of substrate phosphorylation, which mediates the differentiation or maturation of hematopoietic cells [3].

In conclusions, demonstration of increased tyrosine phosphatase activity for phosphopeptide-1 in patients with acute leukemia compared to the control group, as evaluated in our study, suggests that the significant increase in tyrosine phosphatase activity may play a role in leukemogenesis. Studies on a larger scale involving a larger patient population would clearly demonstrate this effect and may further draw atten-

tion to the use of tyrosine phosphatase in the treatment of patients with leukemia.

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