Lysosomal Arylsulfatases of Human Leukocytes: Increment of Phosphorylated B Variants in Chronic Myelogenous Leukemia

Yoshio Uehara, Shinsei Gasa, Akira Makita, Keisuke Sakurada, and Tamotsu Miyazaki

Biochemistry Laboratory, Cancer Institute [S. G., A. M.] and Third Department of Internal Medicine [Y. U., K. S., T. M.], Hokkaido University School of Medicine, Sapporo 060, Japan

INTRODUCTION

Lysosomal arylsulfatases A and B of peripheral leukocytes from patients with chronic myelogenous leukemia and from healthy subjects were studied. Two enzyme activities of leukemia cells were significantly higher than those of cells from healthy subjects, irrespective of total and differential counts of leukemic cells. Upon anion-exchange chromatography, the arylsulfatases of chronic myelogenous leukemia cells and normal leukocytes were separated into the basic B enzyme and its anionic variant (B\(_i\)) and A enzyme. However, the amount of B\(_i\) enzyme relative to B enzyme or the activity ratio of B\(_i\) enzyme to total arylsulfatase B (B + B\(_i\)) was higher in chronic myelogenous leukemia cells than in normal cells.

The anionic property of the enzyme was found to be due to phosphate groups bound to the carbohydrate moiety of the arylsulfatase, based on the following results. When B\(_i\) enzyme was treated with alkaline phosphatase followed by isoelectrofocusing, it was changed to a less anionic enzyme with heterogeneous components which are ascribed to phosphodiester groups linked to the heterogeneous carbohydrate moiety of the enzyme; no effect was observed by sialidase treatment. Upon treatment of B\(_i\) enzyme with endo-\(\beta\)-N-acetylglucosaminidase H, which cleaves sugar chains of a high mannose type in glycoproteins, the anionic heterogeneous components were converted to the basic component similar to B enzyme.

From our present and previous observations, it can be concluded that the increase of phosphorylated forms of the lysosomal hydrolase represents one characteristic of rapidly proliferating neoplastic cells.

MATERIALS AND METHODS

Chemicals. Potassium salts of p-nitrocatechol sulfate and protease-free neuraminidase from Arthrobacter ureafaciens were purchased from Nakarai (Kyoto, Japan), alkaline phosphomonoesterase (type III) of Escherichia coli from Sigma Chemical Co. (St. Louis, Mo.), and endoglycosidase H of Streptomyces griseus from Seikagaku Kogyo (Tokyo, Japan). Other chemicals were of reagent grade.

Patients. Diagnosis of CML was based on clinical, morphological, and biochemical features: chronic phase of disease; splenomegaly; WBC \(>30,000\) per \(\mu\)l; elevated enzyme activity levels; and the presence of Ph\(^1\) chromosome and low alkaline phosphatase score in peripheral neutrophils. No clinical sign of infection was observed in any patient at the time of study.

Preparation of Normal Leukocytes and Leukemic Cells. Heparinized venous blood was obtained from normal volunteers and patients with CML. Leukocytes from healthy volunteers and CML patients were separated from freshly drawn heparinized blood by sedimentation using dextran (M, 177,000) as described by Kolodny and Mumford (19). Cells were washed with 0.25 m sucrose in 10 m Tris-HCl buffer (pH 7.5) and stored at \(-80^\circ\) until analysis.

Preparation of Arylsulfatases. This was carried out in a manner similar to that of the previous procedures (14). Briefly, cells were homogenized by a Brinkmann homogenizer, followed by centrifugation for 10 min at 500 \(\times\) g. The supernatant was then saturated with ammonium sulfate to 75%, and the resultant precipitate was dialyzed and applied on a DEAE-cellulose column equilibrated previously with 10 m Tris-HCl, pH 7.5.

Enzyme Assay. Arylsulfatases A and B activities were separately assayed using p-nitrocatechol sulfate as a substrate by the method of Baum et al. (2), with a slight modification. For the assay of the combined arylsulfatase A and B activity, 10 m p-nitrocatechol sulfate was used at a final concentration in 0.25 m sodium acetate buffer, pH 5.6 (17). One unit of arylsulfatase is the enzyme amount producing 1 nmol of p-nitrocatechol per hr. Protein was determined according to the method of Lowry et al. (21).

Isoelectric Focusing on Polyacrylamide Gel. This was carried out similarly to the previous procedure described by Stevens et al. (33) in a pH range of 3.5 to 10 at 4\(^\circ\) for 4 hr, with a potential gradient of 200 to 700 V. The enzyme activity on the gel was visualized by staining with p-nitrocatechol sulfate (15).

Neuraminidase Treatment. The preparations of arylsulfatases B and B\(_i\) (40 units each) were incubated with Arthrobacter neuraminidase (0.1 units).
units) in 0.1 M phosphate buffer, pH 6.2, at 37° for 3 or 16 hr at a final volume of 0.3 ml. Control experiments were performed under similar conditions without the addition of the neuraminidase.

Phosphatase Treatment. The arylsulfatase preparation (40 units) was incubated with alkaline phosphatase (10 milliunits) in 0.1 M Tris-HCl buffer, pH 8.2, for 3 or 16 hr at a final volume of 0.3 ml. To assay endogenous phosphatase activity possibly included in the partially purified arylsulfatase preparation, the preparation (40 units) was incubated with 6.6 mM p-nitrophenyl phosphate as a substrate in a final volume of 1 ml at pH 5.0 (0.25 M sodium acetate buffer). After incubation at 37° for 30 min, the reaction was terminated by the addition of 1 ml of 1 M NaOH, followed by colorimetric assay at 410 nm for the amount of p-nitrophenol liberated.

Endoglycosidase H Treatment. The incubation mixture contained 200 units of arylsulfatase (in 0.3 ml of 10 mM Tris-HCl buffer, pH 7.5) and 25 milliunits of endoglycosidase H (in 0.1 ml of 100 mM sodium citrate buffer, pH 5.5) in a dialysis bag. The mixture was incubated at room temperature for 16 hr under dialysis against 50 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid buffer, pH 6.2. The dialysate was concentrated, dialyzed against 10 mM Tris-HCl buffer, pH 7.5, and subjected to isoelectric focusing.

pH Optimum and Time Course of the Enzymes. pH Optimum of the enzyme preparations (40 units each) from peaks B, B1, and A were separately assayed at various pHs of 0.5 M sodium acetate buffer using 10 mM p-nitrocatechol sulfate without the addition of arylsulfatase inhibitors. The time course profiles of the arylsulfatases (40 units each) were measured at the activity of the various times of incubation by the combined arylsulfatase A and B assay method as described above.

RESULTS

Activities of Arylsulfatases A and B in Leukemic Cells and Normal Leukocytes

The leukemic cells had significantly higher levels of arylsulfatase activities than did those of normal leukocytes (Table 1), and these activities did not correlate with the total and differential counts of leukocytes, neutrophil alkaline phosphatase score, or the degree of splenomegaly. Arylsulfatase B activity was much higher than was arylsulfatase A activity in both the normal and leukemic cells.

Fractionation of Arylsulfatases by Anion-Exchange Chromatography

Chromatography on a DEAE-cellulose column revealed that the arylsulfatases of normal leukocytes consisted of 3 major components (peaks B, B1, and A in Chart 1a). Lymphocytes and granulocytes which were separated from the leukocyte fraction of healthy subjects showed essentially the same chromatographic profiles. CML cells also demonstrated 3 enzyme-active components (Chart 1b). However, the specific activity in each component and the activity profile of the B1 relative to the B component were considerably higher compared to those of normal leukocytes. Although some variations were observed between individual CML patients, the enzyme profile was essentially the same as that of Chart 1b. Heterogeneous profiles of arylsulfatase B1 and B of normal and leukemic cells imply the charge heterogeneity of the enzymes, and the negatively charged B1 enzyme was increased in CML cells. When the ratio of B1 enzyme activity to the total (B + B1) arylsulfatase B activity was assayed, the ratio of CML cells was markedly higher than that of normal cells (Table 1).

Isoelectric Focusing of Arylsulfatases

The pl of arylsulfatase B (Peak B) either from leukemic cells or normal leukocytes was in an alkaline range (Fig. 1). On the other hand, Peak B1 enzyme from normal or leukemic cells focused at a wide range near pl 8 with considerable heterogeneity. Peak A enzyme had a pl of about 5.0 and was distinguished clearly from the Peak B or Peak B1 enzymes.

Properties of Arylsulfatases of Leukemic Cells and Normal Leukocytes

pH Optimum. The pH optimum of Peak A enzyme from leukemic cells or normal leukocytes was approximately 4.6, while...
both the Peak B and Peak B₁ enzymes from leukemic cells and normal leukocytes had an optimum pH of 6.3.

**Time Course.** Hydrolysis of the substrate catalyzed by the Peak A enzyme either from leukemic cells or normal leukocytes was not proportional to incubation time, demonstrating a typical anomalous curve which was characteristic of arylsulfatase A (24). On the other hand, both the Peak B and Peak B₁ enzymes demonstrated a linear relationship to hydrolysis versus time.

**Effects of Exogenous Hydrolases on Arylsulfatases B and B₁.**

In order to examine the cause of the negative charge of the B₁ enzyme, the B₁ enzyme preparation was treated with exogenously added neuraminidase and phosphatase, followed by examination with isoelectric focusing (Fig. 2). Upon treatment with exogenous alkaline phosphatase for 3 hr, a portion of leukemic B₁ enzyme was shifted to a cathodic region to give heterogeneous components (Fig. 2, Lane 7) and, for 16 hr, all components of the intact enzyme were changed to several components which moved to a more cathodic region (Fig. 2, Lane 9). The control experiments (at pH 8.5) did not exhibit detectable change, although the B₁ enzyme preparation used contained some endogenous acid phosphatase activity. On the other hand, no effect of exogenous neuraminidase was observed in any arylsulfatase preparations. These results indicate that leukemic B₁ enzyme is phosphorylated but not sialylated. The leukemic Peak B enzyme was also examined by treatment with exogenous hydrolases and found to be phosphorylated (Fig. 3) but not sialylated. Phosphorylation of both the Peak B and Peak B₁ enzymes from normal leukocytes was also demonstrated on the basis of the results similar to those of leukemic enzymes (data not shown). The leukemic B₁ enzyme was treated with endoglycosidase H, which cleaves carbohydrate chains at the chitobiose unit of most glycopeptides with high mannose-type structure, followed by isoelectric focusing. As shown in Fig. 4 (Lane 2), the heterogeneity detected in B₁ enzyme almost completely disappeared upon the treatment and shifted to the alkaline range, similar to the untreated B enzyme.

The above results clearly indicate that the arylsulfatase B variant (B₁) which was increased in CML cells was phosphorylated on its carbohydrate moiety.

**DISCUSSION**

In the present study, we demonstrated significantly elevated activities of both the arylsulfatases A and B in human myelogenous leukemic cells compared to leukocytes from healthy subjects. Elevated activities of these enzymes were also found in the urine of the patients with myeloid leukemia (9, 27) and with acute nonlymphocytic leukemia (25). However, such enzymic elevation in leukocytes or urine does not seem common to all types of leukemia. On the contrary, in chronic lymphocytic leukemic cells, enzyme levels were rather decreased (20, 36). Since the activity levels of arylsulfatases differ from one leukocyte class to another [in decreasing order of eosinophil, basophil, granulocyte, and lymphocyte (36)], the levels observed in different leukemic cell types appear to reflect the their progenitors, being increased in myeloid leukemic cells and decreased in lymphoid leukemic cells.

Anionic arylsulfatase B variants have been noted in human fibroblasts (32), brain (34), kidney (17), rat eosinophils (37), rat basophil leukemia tumors (37), and rat mast cells (25), although the acidic nature was not elucidated. The activity and kinetic properties of arylsulfatases from human leukocytes with metachromatic leukodystrophy, a genetic arylsulfatase A deficiency, were examined (8, 16, 28, 29). However, the molecular properties of arylsulfatases isolated from human leukocytes either from healthy subjects or patients have not been demonstrated.

In the present study, arylsulfatase B from either CML cells or normal leukocytes was shown to be very charge-heterogeneous. The amount of an anionic variant B₁ was markedly increased in CML cells. Similarly, the occurrence of anionic variants of lysosomal β-hexosaminidase and other glycosidases was also observed in acute lymphocytic leukemia cells (3, 5, 7, 12). As to the respective glycosidases, the activity of the variants relative to the total activity correlated with patients’ clinical status, increasing during progression of the diseases and decreasing toward normal activity after therapy (3, 5, 12). These anionic variants were suggested to be due to sialylation of β-hexosaminidase enzyme (5). In our previous results, the B₁ variant isolated from human lung cancer was shown to be modified by sialylation as well as by phosphorylation of the enzyme (15). However, the variants of CML cells were not susceptible to sialidase, suggesting varying carbohydrate structures of enzyme from different sources. On the other hand, lysosomal hydrolases having high mannose-type oligosaccharides have been demonstrated to undergo a posttranslational processing on the nonreducing termini at their carbohydrate moiety (18), with sequential formation of phosphodiester, phosphomonoester, and mature dephosphorylated forms (35). Arylsulfatase B was reported to be a glycoprotein with high mannose-type sugar chains (1). Therefore, the increase of the B₁ enzyme, which is phosphorylated on its carbohydrate moiety, may represent a tumor-associated impairment in the above processing.

On the other hand, we recently found that the anionic B₁ variant of human lung cancer was also phosphorylated on the protein moiety as well as on the carbohydrate moiety (13). It seems likely, therefore, that some components, if not all, of the CML anionic B₁ enzyme are also phosphorylated on the protein moiety, and studies along this line are in progress. Elevated protein kinase activity in human myeloblastic cells (11) may be attributable to protein phosphorylation of arylsulfatase B enzyme of CML cells.

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**REFERENCES**


Arylsulfatases in Human Leukocytes


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Fig. 1. Isoelectric focusing patterns of arylsulfatases from normal leukocytes and leukemic cells. The enzyme preparations after DEAE chromatography were isoelectricfocused on polyacrylamide gels in a pH range of 3.5 to 10, and the gels were stained for the enzyme activity as described in "Materials and Methods." Lane 1, arylsulfatase A from normal leukocytes; Lane 2, arylsulfatase B from normal leukocytes; Lane 3, arylsulfatase B from leukemic cells of CML; Lane 4, arylsulfatase B from normal leukocytes, including hemoglobin (Hb) as a marker; Lane 5, arylsulfatase B from leukemic cells.

Fig. 2. Effects of neuraminidase and phosphatase on B enzyme preparation from leukemic cells. Bs, B enzyme preparation, after DEAE chromatography, from leukemic cells. Lane 1, incubated with neuraminidase; Lane 2, incubated with neuraminidase and phosphatase; Lane 3, incubated with neuraminidase and phosphatase; Lane 4, incubated with neuraminidase, phosphatase, and neuraminidase.

Fig. 3. Effects of phosphatase on B enzyme preparation from leukemic cells. The B enzyme preparation from leukemic cells of a CML patient was incubated at pH 8.5 at 37° with or without exogenously added phosphatase, isoelectricfocused on polyacrylamide gels in a pH range of 8 to 9.5, and stained for arylsulfatase activity as described in "Materials and Methods." Lane 1, incubated without exogenous phosphatase for 3 hr; Lane 2, treated with alkaline phosphatase for 3 hr; Lane 3, incubated without phosphatase for 16 hr; Lane 4, incubated with alkaline phosphatase for 16 hr.
Fig. 4. Effect of endoglycosidase H treatment on leukemic B̄ enzyme. B̄ enzyme from CML cells was treated with endoglycosidase H, followed by isoelectric focusing in a pH range of 3.5 to 10, as described in "Materials and Methods." Lane 1, nontreated B̄ enzyme from CML cells; Lane 2, B̄ enzyme treated with endoglycosidase H; Lane 3, nontreated B̄ enzyme from CML cells. Hb, hemoglobin as a marker.
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