Alkaline Phosphatase Activity of Normal and Malignant Human Lymphocytes

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Abstract Bone marrow aspirates from patients with multiple myeloma were obtained and lymphocytes were isolated using density gradient centrifugation. The APase activity of the samples ranged from 12 to 88 nmol/0.2 × 10⁶ cells as compared to the very low activity present in normal human peripheral blood lymphocytes PBL. Neither the unstimulated nor the mitogen stimulated lymphocytes from peripheral blood of normal humans showed appreciable APase activity. The lymphocytes from bone marrow of multiple myeloma patients as well as myeloma cell lines—RPMI 8226 and U266 B1 express APase activity constitutively. The results of the present investigation are discussed in the light of existing literature.

Keywords Alkaline phosphatase · Human peripheral blood lymphocytes · U266B1 and RPMI 8226

Introduction

Enhancement of APase activity has been demonstrated to be an integral feature of B cell activation and differentiation. In an activated B cell, probably, it aids in the metabolite transport to fulfill the demands of the growth and proliferation. Although many other functions have been attributed to APase, its role in malignancy needs to be evaluated. A change in the level of serum APase has been

used as an important diagnostic marker in several disease states. APase activity provides the clinician, valuable information for diagnosis and follow up of patients during treatment. APase isozyme patterns have been reported in malignancies and renal diseases [1]. Tumorigenicity has been correlated with high levels of APase expression [2]. Human APases are abundantly expressed in tumor cells, and serum levels of APase isozymes are often valuable tumor markers [3].

Increased level of APase activity has been reported in neutrophils in chronic myeloproliferative diseases [4]. Hemopoietic tumor cell lines have been shown to express high APase activity [5]. Especially in B lymphoid cell lines APase activity has been reported to be high. APase activity has been demonstrated in several murine tumor cell lines of B lymphoid lineage and in few human lymphoid cell lines [6]. Thus, expression of APase is considered as one of the important identifiable markers of malignancy. Various tumors and tumor cell lines such as teratocarcinomas, choriocarcinomas and osteosarcomas express APase activity [7]. In the present study, normal human peripheral blood lymphocytes and mononuclear cells from bone marrow of multiple myeloma patients were screened for the presence of APase activity. Also two human myeloma cell lines RPMI 8226 and U266 B1 were screened for APase activity and for preliminary characterization of the enzyme.

Materials and Methods

Cell Lines and Culture Conditions

U266B1 and RPMI 8226 cell lines were obtained from National Center for Cell Science, Pune, India. They were

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maintained in RPMI-1640 (Sigma-Aldrich, India) tissue culture medium supplemented with L-glutamine, and 10% heat inactivated Fetal Calf Serum (Biological Industries, S.A.) in 25 cm² tissue culture flasks and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

³H-Thymidine Incorporation Assay

Cells were cultured in triplicate in 200 µl of complete medium RPMI 1640 in 96 well flat bottomed microtitre plates. Cultures were kept at 37 °C in a humidified incubator with 5% CO₂. The cultures were pulsed with 0.5 µCi of ³H-thymidine for the last 24 h of the culture period and were harvested on to glass fibre filter using Skatron automatic cell harvester. The dried filters were transferred into toluene based scintillation cocktail and the radioactivity was measured using Beckman scintillation counter.

Assay of APase Activity

Alkaline phosphatase activity was determined by p-NPP hydrolysis. The absorbance of p-nitro phenol (p-NP) produced was measured at 405 nm. Cells were dispensed into wells of microtitre plate and centrifuged at 450 g for 10 min at 4 °C. The cells were suspended in 0.9% saline and the centrifugation step was repeated. To the pellet 180 µl of 1 mg/ml p-NPP in 0.1 M bicarbonate buffer with 2 mM MgCl₂ was added. Incubation was carried out at 37 °C in a humidified incubator. After 30 min the reaction was stopped by the addition of 20 µl of 1 N NaOH and the absorbance was measured at 405 nm using ELISA plate reader. The amount of p-NP released was calculated from a standard graph. The results were expressed as nmol of p-NP released/0.2 × 10⁶ cells.

Isolation and Mitogen Induced Proliferative Response and APase Activity of Human Peripheral Blood Lymphocytes (PBL)

PBLs were isolated from venous blood collected into heparinised tubes (10 µ/ml). The blood was diluted 1:1 with saline and 8 ml of it was layered over 3 ml histopaque and centrifuged at 500 g for 20 min. The cells at the interface were collected and washed thrice with complete medium and suspended in the same medium. Human PBL were cultured with the indicated concentrations of Pokeweed mitogen (PWM) and the proliferative response was measured using ³H-Thymidine incorporation into DNA. The cultures were assessed for ³H-Td incorporation and APase activity was estimated at the end of the culture period.

Screening of Lymphocytes from Bone Marrow Samples of Multiple Myeloma Patients

The bone marrow aspirate samples were obtained from Department of Pathology, Nizam's Institute of Medical Sciences, Hyderabad. The sample was a leftover sample of aspirate taken for diagnostic purpose. No special ethical clearance was obtained as the samples were primarily taken for diagnosis. The sample was kept in RPMI 1640 at 4 °C till they were processed. The samples were first brought to room temperature and then were dispensed by gentle aspiration using a Pasteur pipette. The clumps were allowed to settle down for 5 min and the supernatant cell suspension was collected and lymphocytes were isolated and assayed for APase activity.

Fluorescence Immuno Staining of Cells

RPMI 1640 medium containing 0.05% sodium azide is used during staining for washing cells. Human lymphocyte culture/myeloma cells (one million cells) were centrifuged at 400g for 10 min and the cell pellet was washed twice with 1 ml of medium. To the cell pellet, 5 µg of antibody to human APase was added and incubated for 1 h at 4 °C. The cells were washed twice with medium to remove the unbound antibody by centrifugation. To the cell pellet, 10 µl of 1:10 diluted goat anti mouse IgG FITC conjugated antibody was added and incubated for 1 h at 4 °C. The cells were washed twice with medium to remove the unbound FITC conjugated antibody. The cells stained for APase were analysed by fluorescence microscopy.

Cytochemical Staining of Lymphocytes for APase Activity

Myeloma cells were centrifuged at 200 g and the pellet was washed with saline. To the pellet BCIP/NBT in Tris buffered saline was added and incubated for 10 min at 37 °C in humidified incubator. The cells were washed thrice with saline to remove the excess substrate and the cells positive for APase were observed using an inverted microscope.

Activity Staining of Enzyme on Polyacrylamide Gel

Cell suspension was harvested by centrifugation at 400 g for 10 min. The cells were washed twice with saline and then suspended in 10 mM Tris buffer containing 2 mM MgCl₂ pH 7.4. The cells were sonicated at half-maximal amplitude for 15 s for 15 cycles with 30 s interval. To the sonicate, TX-100 was added to a final concentration of 0.5% and centrifuged at 15,000×g for 20 min. The supernatant was collected and loaded on a 7.5% Native polyacrylamide gel and electrophoresis was carried out at a constant voltage of 60 V for 2 h. The gel was then

incubated in APase substrate solution BCIP/NBT. The reaction was terminated by washing the gel with water [8].

Determination of K_m and Effect of Inhibitors

All the assays were carried out in triplicates. Substrates blank were included in each experiment to measure the non enzymatic hydrolysis of substrate. For the determination of K_m , the reaction rate was measured at the following substrate (pNPP) concentrations 2.5 mM, 5.0 mM, 10 mM, 20 mM. The data was then fitted to the Michaelis–Menten equation using non linear regression to find K_m values. APase activity was estimated to be higher in diethanolamine buffer as compared to bicarbonate buffer, pH 10.0. Hence, for kinetic experiments, diethanolamine buffer was used. The substrate, p-NPP was prepared in 0.10 M Diethanolamine buffer, pH 10.0 with 5 mM $MgCl_2$. OD was taken at 405 nm after 1 h incubation at 37 °C. A Line weaver-Burk plot, $1/[V]$ Vs $1/[S]$ was plotted. The value of X-intercept was used to calculate K_m . The formula, X-intercept = $-1/K_m$. The enzyme activity was measured in the presence of varying concentrations of the inhibitors—levamisole, L-homoarginine, L-phenyl alanine and theophylline. To determine K_i , APase activity was measured as residual activity compared to the activity in the absence of inhibitor, and the inhibition constants were calculated by non linear regression analysis.

Results

Mitogen Induced Proliferative Response and APase Activity of Human Peripheral Blood Lymphocytes

Human PBLs were cultured with and without mitogens, PWM (B cell specific), for 72 h. The results show that a significant increase in the proliferative response was observed with the mitogen in a dose dependent manner Fig. 1a. However, no enhancement of APase activity was detectable (Fig. 1b).

APase Activity of Lymphocytes Isolated from Bone Marrow Samples of Multiple Myeloma Patients

Bone marrow aspirates from patients with multiple myeloma were obtained and lymphocytes were isolated using density gradient centrifugation. The APase activity of the samples ranged from 12 to 88 nmol/0.2 $\times 10^6$ cells as compared to the very low activity present in normal human PBL. The results are presented in Table 1.

Fig. 1 Proliferative response and APase activity of mitogen stimulated normal human peripheral blood lymphocytes. Values represented are mean $\pm$ SEM of three experiments. *Values significantly differ from the respective control, $p < 0.05$

Table 1 APase activity of B cells isolated from human peripheral blood and from bone marrow aspirates of multiple myeloma patients

Sample	APase activity (nmol/0.2 $\times 10^6$ cells)
B cells from peripheral blood	3.55 $\pm$ 0.77 ^a
Myeloma bone marrow cells ^b	
Sample 1	12.50 $\pm$ 0.07
Sample 2	87.90 $\pm$ 4.97
Sample 3	12.30 $\pm$ 2.72
Sample 4	20.30 $\pm$ 1.20
Sample 5	13.80 $\pm$ 4.40

^aValues presented are mean $\pm$ SEM of five samples

^bValues presented are mean $\pm$ SEM of triplicates

Screening of Cell Lines for APase Activity

Based on the above observations, two human cell lines were procured from National Centre for Cell Sciences, Pune, India. RPMI 8226 and U266 B1 myeloma cell lines. SupT1 a human T cell lymphoma cell line were screened

for APase activity. The results are presented in Table 2. It is observed that both the human myeloma cell lines had significant APase activity which is constitutive.

Immunofluorescence Analysis of Mitogen Stimulated Normal Human Peripheral Blood Cells and Myeloma Cell Lines for APase

The above observations demonstrated the presence of APase in myeloma cell lines based on the measurement of catalytic activity. Indirect immunofluorescence analysis was carried out to detect the presence of the enzyme protein on the membrane using a monoclonal antibody to human bone APase. The unstimulated human peripheral blood lymphocytes and PWM stimulated human peripheral blood lymphocytes were treated with the unconjugated monoclonal antibody (mouse) for human APase followed by the FITC conjugated anti-mouse Ig G1 secondary antibody. The results show that unstimulated and mitogen stimulated human PBL were negative for the presence of membrane APase (Fig. 2a–d). On the other hand, U266 B1, human myeloma cells were positive for surface APase (Fig. 3a, b).

Cytochemical Staining

Myeloma cell line, U266 B1 was incubated with BCIP/NBT as substrate in Tris-buffered saline to visualize the presence of APase activity. SupT-1 cells, a T cell lymphoma which were known to have no APase activity were used as a control for comparison. The results are presented in Fig. 4a, b. U266 B1 cells were stained positive for APase activity and the staining was uniform in all the cells. SupT1 cells did not show any staining indicating the absence of the enzyme activity. These results suggest the presence of catalytically active enzyme on the membrane of the intact myeloma cells.

Activity Staining of APase on Polyacrylamide Gel

Further biochemical analysis was carried out using lysates of myeloma cell line U266B1 and RPMI 8226. The lysates were electrophoresed under non-denaturing conditions on a native polyacrylamide gel and was stained with substrate BCIP/NBT. The results are presented in Fig. 5a, b. Both lysates showed significant enzyme activity.

Kinetic Characterization

Preliminary kinetic characterization of APase activity was carried out using U266 cells. Apart from bicarbonate buffer, diethanolamine (DEA) buffer was used to determine activity. The specific activity in DEA buffer was higher (26 nmol/0.2 × 10⁶ cells) as compared to that obtained with bicarbonate buffer (9.7 nmol/0.2 × 10⁶ cells). In all further experiments on characterization of APase, DEA buffer was used. Lineweaver–Burk plot analysis of the data using p-nitrophenyl phosphate as substrate showed that K_m value was 3.57 mM (Fig. 6a). The apparent K_i was determined in the presence of varying concentrations of three inhibitors—levamisole, L-homoarginine, and theophylline. Levamisole was found to be a potent inhibitor of APase with an apparent K_i of 22 μM (Fig. 6b). The inhibition constants of other inhibitors used is presented in Table 3. To determine the type of inhibition by levamisole the enzyme velocity was measured at single concentration of substrate pNPP (2.5 mM) and with varying concentrations of levamisole. The kinetic plot indicates that the inhibition observed with levamisole is of uncompetitive type (Fig. 6c).

Table 2 APase activity of human cell lines

Cell line	Cell conc (× 10 ⁵ cells)	APase activity (nmol/30 min)
RPMI 8226 ^a	0.5	3.25 ± 0.23
	1.0	7.30 ± 0.33
	2.0	13.5 ± 0.1
U266B1 ^a	0.5	4.5 ± 0.01
	1.0	8.0 ± 0.02
	2.0	15.0 ± 0.64
SupT1 ^b	ND	ND

Values represented are mean ± SEM of triplicates

ND not detectable

^aMyeloma

^bT cell lymphoma

Fig. 2 **a, b** Indirect immunofluorescence analysis of unstimulated human PBL for surface APase. **a** Phase contrast ($\times 40$), **b** fluorescence ($\times 40$). **c, d** Indirect immunofluorescence analysis of PWM

stimulated human PBL for surface APase. **c** Phase contrast ($\times 40$), **d** fluorescence ($\times 40$)

Discussion

Distribution of APase is ubiquitous amongst different cell types and tissues in almost all living organisms. Some human tumor cell lines (HeLa) showed an enhanced expression of Placental Alkaline phosphatase [9]. Also, high Tissue-nonspecific alkaline phosphatase activity was detected in cell lines derived from human testicular germ cell tumors. In some choriocarcinoma cell lines the transformation of normal to malignant trophoblast is associated with a switch in expression from Placental Alkaline phosphatase to Germ cell Alkaline phosphatase. The ectopic expression of Placental Alkaline phosphatase has been observed in cancer of the lung, ovary, uterus and gastrointestinal tract. Germ cell neoplasms and somatic tumors were found to express Placental Alkaline phosphatase/Germ cell Alkaline phosphatase. Culvernor et al. [10] have demonstrated that significant APase activity is expressed in cell lines of B lymphoid lineage, including Abelson pre-B, B lymphoma and plasma cell tumor lines. In contrast, T lymphoid and non lymphoid hematopoietic

lines had very low activity. Hence, Tissue-nonspecific alkaline phosphatase activity seems to be a useful marker of B lymphoid lineage. Despite these observations, the function of Tissue-nonspecific alkaline phosphatase activity in malignancy remains largely unknown.

It has been demonstrated earlier that mitogenic stimulation of murine B lymphocytes leads to proliferation and concomitant increase in APase activity [11]. The expression of APase in activated murine B cells was identified as a late marker associated with cell proliferation, progression to differentiation and preceded immunoglobulin secretion [12]. Also, it has been observed that the enhancement of APase activity was B cell specific. Stimulation of murine T cells with Con A, a T-cell specific mitogen did not induce alkaline phosphatase activity [13]. In the present study normal human peripheral blood lymphocytes were stimulated with B cell specific mitogens, PWM and the proliferative response and APase activity were measured. Surprisingly, the resting and mitogen stimulated proliferating T and B-lymphocytes from normal humans did not show enhancement in the APase activity. The lymphocytes

Fig. 3 Indirect immunofluorescence analysis of U266 cells for surface APase. **a** Phase contrast ($\times 40$), **b** fluorescence ($\times 40$)

isolated from bone marrow samples of multiple myeloma patients and multiple myeloma cell lines RPMI 8226 and U266B1 were screened for APase activity. Significant activity was found in these malignant B cells. The enhanced expression of APase activity in myeloma cells was confirmed by APase assay, biochemical staining with BCIP/NBT and immunostaining with monoclonal antibody to human TNAPase analyses.

The characterization of the APase activity of myeloma cells in terms effect of different known inhibitors indicated the enzyme belongs to the tissue non-specific isozyme. Earlier experiments on murine B lymphocytes and hybridomas have also shown that the APase present in these cells belongs to tissue non-specific category [14]. Levamisole inhibited the enzyme activity in a uncompetitive manner [15]. The salient findings of this study are neither the unstimulated nor the mitogen stimulated lymphocytes from peripheral blood of normal humans show appreciable APase activity. The lymphocytes from bone marrow of multiple myeloma patients as well as myeloma cell lines—RPMI 8226 and U266 B1 express APase activity constitutively. The characterization of the APase activity of myeloma cells revealed the enzyme to be tissue

Fig. 4 Cytochemical staining for APase activity. **a** Sup T1, **b** U266 B1. Magnification— $\times 40$; BCIP/NBT was used as substrate

Fig. 5 Activity staining of APase. Lane 1: purified intestinal APase (bovine). Lane 2: myeloma cell lysate. **a** U266B1, **b** RPMI 8226

Fig. 6 **a** Lineweaver–Burk plot of APase (U266) activity using *p*-nitrophenyl phosphate as substrate. **b** Inhibition of APase activity of U266 cell lysates by levamisole. **c** Kinetic analysis of inhibition of APase activity by levamisole

Table 3 Inhibition constant (K_i) of APase activity

Inhibitor	K_i (μM)
Levamisole	22
L-Homoarginine	4000
Theophylline	600

non-specific type. Levamisole inhibited the APase activity of myeloma cell lines significantly in an uncompetitive manner.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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