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Analysis of serum placental alkaline phosphatase activity in testicular cancer and cigarette smokers

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Summary. Serum PLAP/PLAP-like enzymes are elevated in testicular cancer patients and also in heavy smokers. Such elevations were measurable with anti-PLAP polyclonal and monoclonal antibodies, and an antigenic heterogeneity was demonstrated in the circulating enzymes. Thus polyclonal antibody revealed more of tumor PLAP-like antigen while a monoclonal antibody H7 detected PLAP of smokers better. PLAP of smokers and PLAP-like enzyme of cancer (seminoma) patients showed similar characteristics with respect to glycosylation pattern, charge and hydrophobicity. By these properties they were differentiated from PLAP of placenta.

Key words: Placental-like alkaline phosphatase – Testicular cancer – smokers

Placental-like alkaline phosphatase (PLAP-like enzyme) has been recognized as a useful marker for testicular cancer, especially for seminoma [14]. However, it should be remembered that serum PLAP levels are increased by cigarette smoking; the incidence of elevation in smokers is reported to be between 46 and 67% [10, 13, 18, 19]. Moreover, a smoking habit in patients with seminoma evidently contributes to positive results in NED populations during follow-up [2, 5, 19]. The origin of the PLAP activity in smokers is assumed to be the lung, in which PLAP activity corresponding to PLAP of placenta (Regan type) was shown [3, 11]. However, PLAP in sera of smokers was shown to be more similar to the PLAP-like enzyme (Nagao type) in its reactivity with anti-PLAP monoclonal antibodies and its sensitivity to L-leucine [10]. The discrepancy is not properly explained, although a possibility remains that the PLAP-like enzyme may be released from another body organ. To search for a means of discriminating serum PLAP activity between seminoma patients and smokers, we investigated the characteristics of these enzymes with respect to antigenicity, glycosylation pattern, charge and hydrophobicity of the molecules.

Materials and methods

Samples

184 serum samples showing elevated PLAP levels with ELISA (see below) were selected from the cases of testicular tumor examined for PLAP at the National Bacteriological Laboratory in 1987–88. 38 sera of heavy cigarette smoking (> 15 cig/d) but otherwise healthy 19 males and 19 females were collected. Sera were also obtained from three pregnant women in their last trimester. Lung tissues were obtained at autopsies from the Department of Pathology, South Hospital, Stockholm. Seminoma tissues were surgical specimens, and placentae from full term pregnancies. The tissues, stored at −20°C, were thawed, homogenized with physiological saline and extracted with n-butanol (1:1) by stirring for two hours at room temperature. The mixture was centrifuged at 10,000 g for 20 min at 4°C. The aqueous layer was collected and dialyzed against 10 mM Tris buffer (pH 7.0) containing 0.5 mM $MgCl_2$.

Enzyme-linked immunosorbent assay (ELISA)

PLAP content in the sera and the tissue extracts was measured by a highly sensitive and specific sandwich ELISA [20]. Protein-A purified rabbit anti-PLAP IgG was coated on 96 well microtiter plates. After washing, standard purified type I PLAP or samples in 100 µl volumes were added. Following overnight incubation at room temperature and washing, 100 µl of horse-radish peroxidase labelled anti-PLAP IgG: rabbit polyclonal or mouse monoclonal (H7) antibody, were added for 2 h at 37°C. After washing, 100 µl of the substrate O-phenylene diamine were added, and the reaction was stopped with 100 µl of 2.5 M H_2SO_4. Absorbance at 490 nm was measured, and serum concentration of PLAP was calculated. Values above 5 µg/l were considered to be positive [20].

Assay for enzymatic activity

Enzyme activity in fractions of lectin affinity chromatography and high performance liquid chromatography was assayed in a system consisting of 1 M diethanolamine buffer (pH 9.8) containing 0.5 mM $MgCl_2$ and 8 mM p-nitrophenylphosphate (Sigma, St. Louis, Mo.). Fractions following heat inactivation (65°C, 10 min) were placed in 96 well microtiter plates, and 100 µl of the substrate were added to each well. After 1–2 h incubation at 37°C (depending on the activity), the absorbance at 410 nm was measured.

Table 1. Patterns of immunoreactivity of PLAP-like enzyme in smoker and testis cancer sera

Reactivity with antibodies	% positive and PLAP level			
	Testis cancer ($n = 184$)		Smoker ($n = 23$)	
	%	μ/l[a]	%	μ/l[a]
Poly (+), H7 (−)	34	36,4	13	43,1
Poly (−), H7 (+)	22	7,4	52	12,4
Poly (+), H7 (+)	44	poly: 11,4[b] H7: 21,8	35	poly: 8,6 H7: 16,8
Total poly (+)	78	30,5	48	18,0
Total H7 (+)	66	16,8	87	13,6

[a] mean of positive PLAP values with antibodies indicated
[b] mean value excluding 3 samples showing extremely high levels (see also Fig. 1)

Lectin affinity chromatography

Tissue extracts and sera were applied on small columns containing 1.0 ml of Concanavalin A (Con A) - Sepharose or Wheat germ Lectin (WGL) - Sepharose 6MB (Pharmacia, Uppsala, Sweden). The gels, equilibrated with 10 mM Tris buffer pH 7.0 containing 0.15 M NaCl and 0.5 mM $MgCl_2$, were washed with the same buffer and eluted with 0.2 M of α-methyl-D-mannoside (Sigma, St. Louis, Mo.) for Con A-Sepharose and 0.05 M N-acetyl-D-Glucosamine (Sigma, St. Louis, Mo.) for WGL-Sepharose. Fractions of 1.5 ml were collected and their catalytic and immunochemical activities were measured.

Isoelectric focusing (IEF)

IEF was performed by an LKB Multiphor apparatus in thin layer polyacrylamide gels (Ampholine PAG plates, LKB, Stockholm, Sweden), pH range 4.0–6.5. To obtain catalytic activity in serum samples comparable to tissue extracts, a sample showing the highest PLAP value with MAb H7 ELISA was selected from patients with testicular tumors or smokers and concentrated by lyophilization, following the butanol extraction procedure. The samples were applied to 5 × mm filter papers placed at the cathode. Focusing was performed at 10°C and with a constant power of 25 W, with 0.1 M glutamid acid in 0.5 M H_3PO_4 as the anodic and 0.1 M β-alanine as the cathodic solution. After focusing for 2.5 h, the gel was submerged in a solution consisting of 0.25 M Tris/maleic acid pH 9.8, 0.8 mM α-naphthyl phosphatase, 1 mM 4-aminodiphenylamine diazonium sulfate and 5 mM $MgSO_4$ for 3 h at 37°C. After rinsing the gel with destaining solution (25% v/v ethanol and 16% v/v acetic acid in water), the IEF pattern was photographed.

High performance liquid chromatography (HPLC)

Tissue extracts and sera were analyzed by a TSK-gel phenyl-5PW column with high performance liquid chromatography (Waters, Milford, Mass.). The separation condition included a 30 min linear gradient from 1 to 0 M of ammonium sulphate in water containing 0.5 mM $MgCl_2$. The flow rate was 1.0 ml/min. Eluate was collected every two minutes and dialyzed against 0.5 mM $MgCl_2$, 10 mM Tris buffer, pH 7.0.

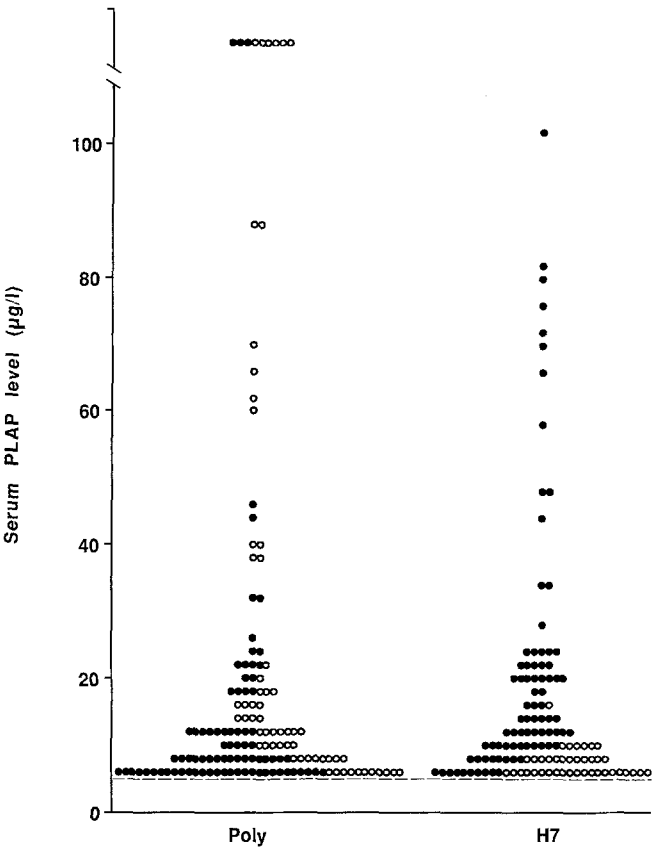

Fig. 1. Serum PLAP levels of patients with testicular cancer. Values with polyclonal (poly) and monoclonal antibody (H7) are indicated. Closed circles represent samples positive with both polyclonal and monoclonal assays. Open cirlces indicate samples only positive with one assay. All values are above the cut-off level of 5 μg/l

Results

Of 184 PLAP positive serum samples from patients with testicular tumors, 62 samples (34%) were positive only with rabbit polyclonal antibody and 40 samples (22%) only with mouse monoclonal antibody (MAb H7) (Table 1). We only analyzed the distribution of sera with values above cut-off in one or both assays. In samples only positive with one assay, most of the elevated values with MAb H7 were only slightly above the cut-off value, while some of the values with polyclonal antibody were considerably higher (Fig. 1). MAb H7 was selected since it was shown to be reactive with all common phenotypes of PLAP as well as the PLAP-like enzyme in extracts of seminomas and normal testes [7, 12, 21]. However, the results indicate an antigenic heterogeneity of the circulating antigen and also that some of the tumor-related PLAP would be missed if a MAb or a polyclonal antibody were used alone for detection. In sera positive with both polyclonal antibody and MAb H7, MAb H7 generally gave the higher values (Fig. 1), indicating that MAb H7 had a strong reactivity with its defined epitope. This is also supported by the fact that 40 samples were positive only with MAb H7. However, the total sensitivity in these cancer sera seemed to be higher (78%) when polyclonal

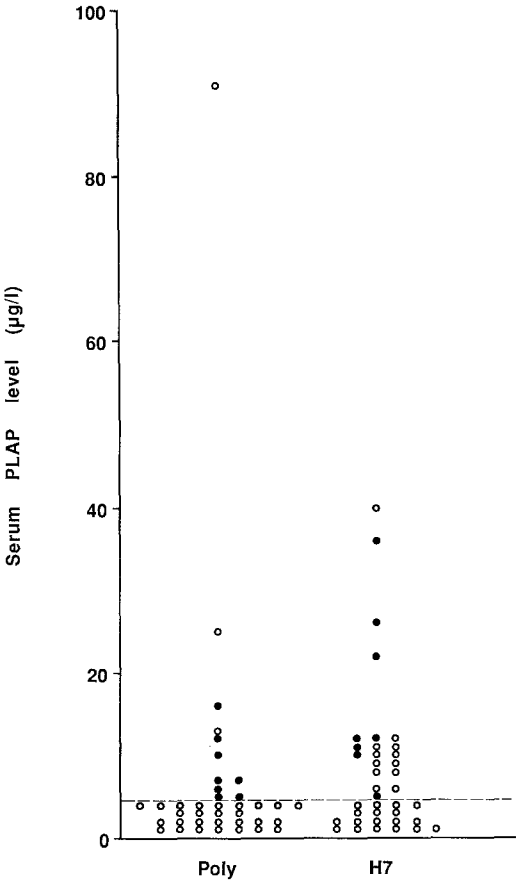

Fig. 2. Serum PLAP levels of smokers. Closed circles represent samples positive with both assays

antibody was employed for detection (Table 1), indicating that the MAb H7 epitope may sometimes be lacking or hidden in the circulating antigen.

Using the same antibodies, the total frequency of elevated serum PLAP levels in smokers was found to be 61% (23/38) (Fig. 2). The distribution of positive sera between sexes was almost equal (men, 10; women, 13). In contrast to the findings with cancer sera, more smoker sera were evaluated as positive with MAb H7 (97%) than with polyclonal antibody (48%) (Table 1). The incidence of MAb H7 negative and polyclonal antibody positive samples was low compared to testis cancer sera. The findings thus indicate that a heterogeneity of the enzyme in sera of smokers exists, although the variation appears to be smaller compared with sera of patients with testicular cancer.

We have found that the glycosylation pattern of the PLAP-like enzyme in seminoma tissue as well as in sera of seminoma patients differs from that of PLAP [9]. It was therefore of interest to compare the glycosylation pattern of serum PLAP of smokers with the patterns of PLAP and the PLAP-like enzyme by lectin affinity chromatography. Table 2 shows the binding affinity of PLAP or the PLAP-like enzyme to Concanavalin A (Con A) and Wheat germ Lectin (WGL) measured with immunochemical reactivity. The distribution of the immunologically defined reactivity corresponded well to the heat-stable catalytic

Table 2. Binding affinity of immunochemically measured PLAP or PLAP-like enzyme to Concanavalin A and Wheat germ Lectin

	Con A-Sepharose[a]		WGL-Sepharose[a]	
	% unbound	% bound	% unbound	% bound
Serum				
Seminoma serum	56	44	65[b]	35[b]
Smoker serum	53	47	52[b]	48[b]
Pregnancy serum	0	100	33[b]	67[b]
Tissue				
Seminoma tissue	41	59	62	38
Lung	23	77	55	45
Placenta	0	100	0	100

[a] Mean values of three samples are presented as percent of immunochemical reactivity with H7 MAb
[b] The distribution patterns are shown in Fig. 3

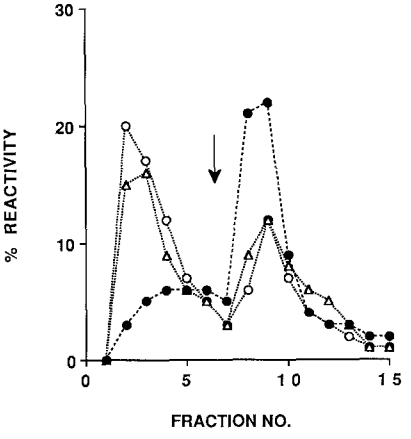

Fig. 3. Binding affinity of circulating PLAP or PLAP-like enzyme to wheat germ lectin. Immunochemical reactivity defined by H7 MAb was measured. Following the unbound fraction, bound fraction was eluted with 0.05 M GlcNAc as indicated by the arrow. Serum of seminoma patient (○), smoker (△), pregnancy (●)

activity. All PLAP activities from pregnancy sera and placentae were bound to Con A, while PLAP in sera of smokers was separated into unbound and bound fractions. The separation pattern of the latter was similar to that of the PLAP-like enzyme from seminoma sera and tissues. In addition, a similar binding property for WGL was disclosed in PLAP of smokers and in the PLAP-like enzyme in seminoma sera and tissues. This property also served to discriminate them from PLAP of placenta, which completely bound to WGL. Although not all activities of PLAP in pregnancy sera were bound to WGL, the separation pattern indicated that its affinity for WGL was stronger than that of the circulating enzymes of seminoma patients or smokers (Fig. 3). Interestingly, the bound and unbound components were also separated in lung tissue with both lectin chromatography techniques.

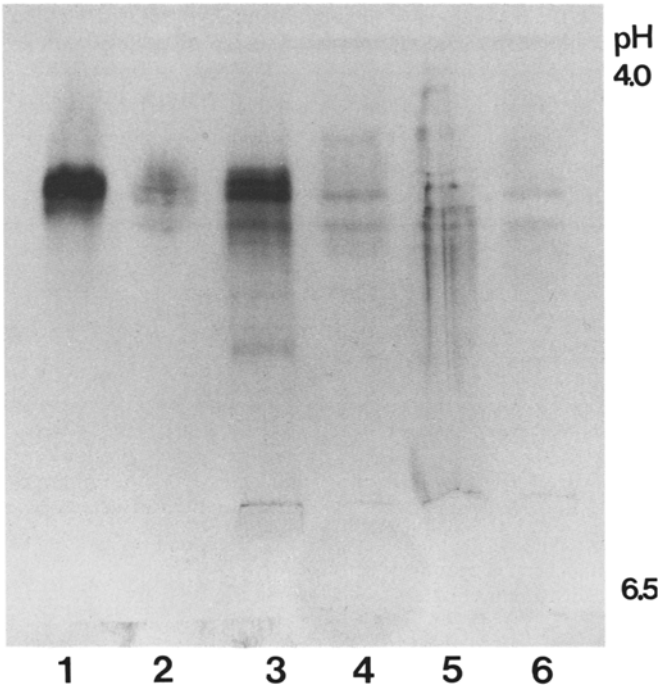

Fig. 4. Enzyme staining after isoelectric focusing. Lane 1, PLAP from placenta; Lane 2, PLAP in pregnancy serum; Lane 3, PLAP-like enzyme from seminoma tissue; Lane 4, PLAP-like enzyme in serum of a seminoma patient; Lane 5, PLAP from lung tissue; Lane 6, PLAP in serum of a smoker

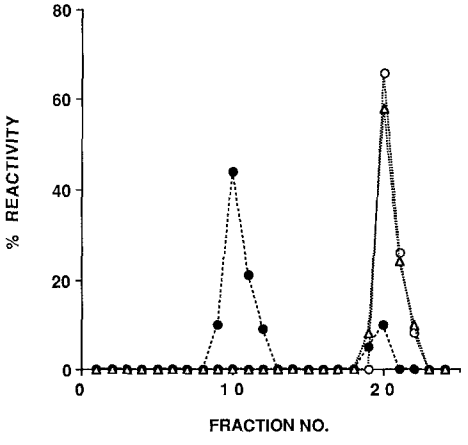

Fig. 5. Distribution of circulating PLAP or PLAP-like enzyme in HPLC separation. Immunochemical reactivity with H7 MAb is indicated. Serum of seminoma patient (○), smoker (△), pregnancy (●)

On isoelectric focusing, PLAP activity in serum of a smoker was visualized on the gel as two bands, corresponding to pIs of 4.5 and 4.6. The staining pattern was very similar to that of the serum PLAP-like enzyme from a patient with seminoma. Enzymes from a seminoma tissue and a lung tissue revealed complex patterns, and bands corresponding to those in sera of a seminoma patient and a smoker could be identified. On the other hand, the main activity of PLAP in placental tissue and in pregnancy serum was stained at pIs 4.4–4.5 (Fig. 4).

A smoker's serum was analysed with HPLC, which permitted differentiation on the basis of hydrophobicity between PLAP in pregnancy serum and the PLAP-like enzyme in serum of seminoma patients (Fig. 5). The PLAP activity of the smoker had the same hydrophobicity as the PLAP-like enzyme of seminoma patients, but differed from PLAP.

Discussion

Discrimination of serum PLAP of smokers from the circulating PLAP-like enzyme in seminoma patients appears to be difficult since the characteristics of these isozymes were shown to be similar as regards glycosylation, charge and hydrophobicity. PLAP activity in lung tissue was reported to correspond to PLAP from placenta (Regan type) with respect to the reactivity with MAbs [11], and migration patterns on starch gel electrophoresis [3]. On the other hand, PLAP in sera of smokers was shown to be the PLAP-like enzyme (Nagao type) according to the reactivity with the same MAbs as above and sensitivity to inhibition with L-leucine [10]. Our observations are compatible with the latter findings, that PLAP of smokers appears to be similar to the PLAP-like enzyme (Nagao type).

The discrepancy reported for the nature of PLAP in sera of smokers and in lung tissue [10, 11] has not been well understood. However, the source of the circulating PLAP in smokers is likely to be the lung, since normal lung contains a significant level of PLAP activity [3, 11, 15] which was immunohistochemically evident in the peripheral respiratory lung parenchyma [15]. In addition, the permeability of the respiratory epithelium was reported to be increased in smokers [6, 8, 16]. Further support comes from our findings of a similar glycosylation pattern in lung PLAP and PLAP of smokers. Recently, PLAP activity in lung tissue has been purified and found to be L-leucine inhibitable [4]. These findings and our data on the glycosylation pattern indicate that the lung contains a PLAP-like substance.

It is well known that cigarette smoking contributes to increased serum CEA levels [1, 17]. This can be explained in the same way as PLAP, because CEA was also shown to be located in the peripheral and the central airways of the normal lung [15]. Actually, 5 out of 38 cases (13%) of smokers investigated showed elevated CEA levels, and interestingly, four of the five (80%) also had increased PLAP levels (unpublished data).

The antigenic heterogeneity of the circulating enzyme seems to be considerable in both smokers and testicular cancer patients. PLAP rises in seminoma patients who are also smokers. This has practical implications for evaluating tumor status of the smoking patients.

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