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Levels of Alkaline Phosphatase Isozymes in Human Seminoma Tissue[1]

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ABSTRACT

The three human isozymes of alkaline phosphatases were quantitatively determined in normal testis and seminoma tissues. The highly selective assays were based on isozyme specific monoclonal antibodies. In the normal testis approximately 90% of the catalytic activity originates from the tissue unspecific alkaline phosphatase, and the remaining activity was due to trace expression of both intestinal (approximately 5%) and placental alkaline phosphatase (PLAP) or PLAP-like isozyme (approximately 5%).

In homogenates of seminoma tissues, highly increased levels of all three isozymes were identified. Both the tissue unspecific alkaline phosphatase and PLAP-like enzymes displayed relative increases of 10- to 100-fold and intestinal alkaline phosphatase 2- to 10-fold compared with normal testis. This finding indicates that the entire genome coding for alkaline phosphatases may be activated in seminomas. The PLAP-like enzyme from seminoma cells comprises a heterogenous population of molecules demonstrating partial heat sensitivity and microheterogeneity upon starch gel electrophoresis in contrast to the pregnancy related PLAP. These findings have implications for the different PLAP assays used in the clinical monitoring of seminoma patients.

INTRODUCTION

The abundance in nature of alkaline phosphatases indicates involvement of fundamental biochemical processes although the physiological function(s) are not clear (1). In humans, at least three gene loci encoding human alkaline phosphatases have been identified, for one of which the complementary DNA has been cloned and sequenced (2, 3). The discovery by Fishman *et al.* (4) that the placental form of the isozyme appeared in the circulation in some malignant conditions was fundamental in oncodevelopmental biology, and placental and placental-like alkaline phosphatase determinations have been widely used to monitor testicular and gynecological tumors (5–7). The serum level of placental alkaline phosphatase in nonpregnant healthy persons is usually very low (8) in contrast to the serum levels of liver-bone-kidney and intestinal alkaline phosphatases which are significantly higher. These isozymes are normally released into the circulation, which may be one reason why their relevance as tumor markers have not attracted much attention. Another reason is the lack of accurate methods to quantify each of these isozymes.

In the normal testis the dominating form (>95%) of alkaline phosphatase is the tissue unspecific (liver-bone-kidney) isozyme with only trace amounts of placental-like alkaline phosphatase present (9). This trace expression of eutopically synthesized placental-like alkaline phosphatase has turned out to be very useful as a tumor marker for malignant tumors of the testis, specifically seminomas, which was recently recognized by the international group report on valuable clinical tumor markers (10).

The introduction of hybridoma technology (11) has enabled the development of highly specific immunochemical tools capable of discriminating in quantitative assays between closely related antigens. Recently, very sensitive immunocatalytical assays based on monoclonal antibodies specific for each of the three isozymes of alkaline phosphatases were developed[3] making it possible to quantify changes in the levels of different alkaline phosphatase isozymes (12). In the present report human normal testes and seminomas were investigated.

MATERIALS AND METHODS

Assays of Alkaline Phosphatase Isozyme

MICA[4] Assays for AP, IAP, and PLAP. MICAs were performed as described by Hirano *et al.* (12).[3] Briefly, Protein-A purified monoclonal antibodies, specific for each isozyme, were covalently coupled to small paper discs (one type of antibody to each set of discs). The discs were incubated with serum or tissue homogenate for 3 h at 37°C. After washing, the paper disc-antibody-antigen complex was incubated for 30 min with substrate solution (sodium phenylphosphate in the case of AP and IAP determinations and 4-methylumbelliferyl phosphate for PLAP). The enzyme reaction was stopped and the absorbance at 500 nm (AP and IAP) or the fluorescence (PLAP) was measured. No heat inactivation was used, and the results obtained are dependent on both intact immunoreactivity and catalytic activity.

Enzyme-linked Immunosorbent Assay for PLAP. An enzyme-linked immunosorbent assay was used as described by Millan and Stigbrand (8) using Protein A purified rabbit polyclonal IgG both as coating antibody and coupled to horseradish peroxidase as a conjugate. The assay was performed in microtiter plates using orthophenylendiamine and H_2O_2 as substrates for the final horseradish peroxidase catalyzed reaction. This assay only measures immunoreactive antigen (PLAP) with no heat inactivation step involved.

Catalytic Assay for PLAP. A sensitive catalytic assay according to Stigbrand *et al.* (13) was used. Heat inactivation of AP and IAP was carried out at 65°C for 10 min before incubation with 25 mM phenylphosphate in 0.05 M carbonate-bicarbonate buffer, pH 9.8, with 5 mM $MgCl_2$ added. This assay measures heat stable catalytically active enzyme only.

Clinical Samples. Venous blood samples from healthy pregnant females attending the gynecological ward for regular medical controls were obtained from Umeå University Hospital. Sera from patients with seminoma were obtained from Karolinska Hospital in Stockholm. Autopsy material and tumor biopsies were obtained from South Hospital and Karolinska Hospital in Stockholm and the Department of Pathology or Forensic Medicine at the University Hospital, Umeå. All samples were kept frozen at −70°C until use. Tissues were homogenized and extracted with butanol before the different assays were performed.

Placental Samples. Placental homogenates of the common phenotypes were selected from a collection of different electrophoretically typed samples. The three common variants of placental alkaline phos-

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[3] K. Hirano, H. Matsumoto, T. Tanaka, Y. Hayashi, S. Iino, U. Domar, and T. Stigbrand. Specific assays for human alkaline phosphatase isozymes, Clin. Chim. Acta, in press, 1987.

[4] The abbreviations used are: MICA, monoclonal immunocatalytic assay; AP, tissue unspecific alkaline phosphatase; IAP, intestinal alkaline phosphatase; PLAP, placental alkaline phosphatase; HCG, human chorionic gonadotropin.

phatase (variants 1, 2, and 2-1) were purified as described by Holmgren and Stigbrand (14).

Starch Gel Electrophoresis. Starch gel electrophoresis and enzymatic staining were performed as described by Beckman *et al.* (15). Common purified phenotypes of placental alkaline phosphatase and homogenates of seminomas with high content of the PLAP-like enzyme were run in two different buffer systems, one alkaline (pH 8.6) and one acid (pH 5.6) in order to differentiate the various phenotypes. After electrophoresis the enzymatic activity of the bands was evidenced by adding a newly prepared solution of 100 mg α-naftylphosphate and 100 mg fast blue RR in 75 mM Tris-5 mM citric acid (pH 8.65) solution. The enzyme reaction was stopped by addition of 35% methanol and 10% acetic acid in water.

RESULTS

The specificity of the assays for each of the isozymes allowed determination of their presence in normal human testicular tissue. As shown in Table 1, all three forms of the isozyme, including the intestinal type, are present. The dominating form is the tissue unspecific AP, which amounts to 0.3–0.6 IU/g tissue corresponding to approximately 90% of the total alkaline phosphatase activity. Both IAP and PLAP-like are eutopically expressed in similar trace amounts in the normal human testis (0.01–0.08 IU/g) (approximately 5% each). The same levels are observed for several nonspecific inflammatory affections or other conditions of the testis like hydrocele, periorchitis, and one hemangioma.

In seminoma cells all three isozymes are significantly elevated. The PLAP content, on the average 3–5 IU/g, corresponds to a relative increase of approximately 100-fold. Significant elevations ($P < 0.001$) are also observed for IAP (0.03–0.4 IU/g) and AP (9–88 IU/g), relative increases amounting to 2 to 10-fold for IAP and 10 to 100-fold for AP, which is the same order of magnitude as that observed for the PLAP-like enzyme. When these levels are compared with the amount of the isozymes in normal liver, intestine, and placenta (Table 2), the levels of AP in the seminoma tissues are higher than those of the normal liver, whereas the absolute levels of IAP and PLAP are lower in the tumors compared with normal small intestine and placenta. These results together indicate a substantial increase in gene expression of all phosphatase isozymes in seminomas, most apparent for AP and PLAP.

The placental form of the enzyme has, as HCG, been established as a useful serum marker for pure seminomas, and a variety of methods for the determination of PLAP have been described (1). Three different assays using different properties of the PLAP-like enzyme were used to compare the levels of enzyme in both normal and seminoma testis tissue (Table 3). The results obtained confirm significant increases with all three types of assays (catalytic, immunocatalytic, and immunochemical), and the data indicate heterogeneity when enzyme activities are compared to enzyme protein concentrations. Pregnancy

Table 1 *Content of alkaline phosphatase isozymes (AP, IAP, and PLAP), in normal and pathological testis tissues measured by MICA*

Samples	Alkaline phosphatase activity (IU/g tissue)		
	AP	IAP	PLAP
Normal testis	0.3	0.08	0.03
	0.6	0.03	0.04
	0.6	0.04	0.03
	0.6	0.01	0.04
Hydrocele	0.4	0	0.05
Periorchitis	0.5	0	0.01
Hemangioma	0.5	0	0.03
Seminoma	88	0.10	2.7
	16	0.04	2.6
	34	0.14	5.5
	13	0.15	2.3
	12	0.08	2.2
	14	0.03	0.9
	20	0.08	3.2
	45	0.27	5.8
	22	0	0.3
	24	0.21	4.3
	39	0.14	3.3
	32	0.43	3.2
	35	0.22	2.6
	54	0.27	3.1
	64	0.32	3.5
	33	0.39	3.4
	9	0.12	1.5
Choriocarcinoma + seminoma	30	0.26	2.4
Choriocarcinoma	0	0	0.03
Yolk sac tumor	6.9	0.72	0
	1.6	0.28	0.02

Table 2 *Levels of alkaline phosphatase isozymes (AP, IAP, and PLAP), in normal human tissues measured by MICA*

Tissue	Alkaline phosphatase activity (IU/g tissue)		
	AP	IAP	PLAP
Liver	0.9	0.03	0.005
	0.4	0.14	0.017
	1.1	0.007	0.002
	1.3	0.004	0.002
	2.6	0.05	0.005
Intestinal mucosa	1.2	70.5	0.18
	0.2	10.0	0.05
	0.5	51.6	0.58
	0.5	19.2	0.34
Placenta	0.14	0.05	24.5
	0.12	0.06	24.4
	0.18	0.06	158.5

Table 3 *Content of PLAP in testis and various testis tumors measured by three different assays*

Sample	MICA (IU/g)	Catalytic assay (IU/g)	Enzyme-linked immunosorbent assay (μg/g)
Normal testis	0.03	0.03	0.05
	0.04	0.03	0.18
	0.03	0.02	0.26
	0.04	0.02	0.26
Seminoma	2.70	1.15	8.4
	2.64	1.41	2.4
	5.53	1.64	19.6
	2.30	0.35	2.1
	2.24	0.96	2.2
	0.90	0.45	1.3
	3.15	1.19	7.9
	5.76	2.68	31.6
	0.33	0.18	0.6
	4.29	1.44	10.3
	3.31	1.13	8.2
	3.17	2.00	10.8
	2.56	1.22	5.3
	3.08	1.05	7.2
	3.49	3.10	19.8
	3.42	4.78	19.0
	1.54	1.40	2.9
Choriocarcinoma + seminoma	2.36	1.46	6.5
Choriocarcinoma	0.03	0.04	0.04
Yolk sac tumor	0	0.15	0.08
	0.02	0.06	0.03

Table 4 *Content of placental alkaline phosphatase in serum from seminoma patients determined by two different assays*

Sample	MICA (IU/liter)	Catalytic assay (IU/liter)
1	12.94	4.92
2	5.46	2.41
3	2.48	1.82
4	2.51	1.17
5	1.42	0.60
6	1.38	0.86
7	0.56	0.41
8	2.63	1.28
9	0.75	0.45
10	1.67	1.03

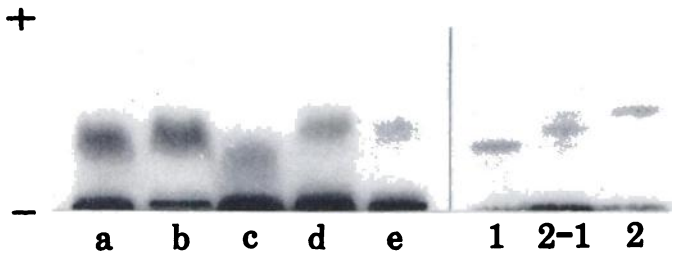

Fig. 1. Starch gel electrophoresis (pH 5.6) of placental alkaline phosphatase in seminoma homogenates (*a–e*) after heat treatment at 65°C for 10 min. The gel was stained for enzymatic activity. To the right of the gel the normal purified phenotypes (*1*, *2-1*, *2*) of placental alkaline phosphatase are applied.

sera assayed in the same way by the three methods (results not shown) display a more homogeneous population of enzyme molecules. In sera from seminoma patients (Table 4) enzyme activities assayed by the MICA and the catalytic assays are variable, in agreement with data obtained from the seminoma tissues. It should furthermore be concluded that in pregnancy sera the MICA and catalytic assays give almost identical values, but in tumor sera the MICA results in 2-fold higher values. It is concluded from these observations that the tumor associated PLAP-like form of the enzyme is partially heat sensitive compared with the pregnancy related PLAP.

Some of the homogenates demonstrating high content of PLAP were subjected to starch gel electrophoresis after heat treatment as shown in Fig. 1, and the phenotypic expression was compared with that of the three common purified phenotypes. It can be concluded that the tumor associated isoforms are polymorphic, but the patterns cannot be allocated to any of the normal placental types. A considerable microheterogeneity is also observed with broad bands for all samples investigated.

When all the phosphatase isozymes, used as markers, were compared with the levels of α-fetoprotein and HCG in the tumors (results not shown) α-fetoprotein was significantly elevated in the yolk sac tumors and not detected in any other conditions, whereas HCG content was significantly elevated in the choriocarcinomas and elevated in 4 of the 17 seminomas. Interestingly, the highest levels of IAP in these groups were observed in the yolk sac tumors in agreement with the endodermal origin of these tumors.

DISCUSSION

One of the basic concepts in oncodevelopmental biology has been the similarities in enhancement of specific gene expression during both the normal embryogenesis and malignant transformation causing rapid growth. This process generates an increased synthesis of growth promoting cell components which are typical and often identical in both the tumor and the fetal cells. A number of such oncofetal or carcinoembryonic antigens have been described, one of which is the placental alkaline phosphatase (16). This isozyme is detectable in normal human sera during pregnancy and certain malignant conditions, and the identification is simple because of the low levels of PLAP in a normal healthy population and the access to highly sensitive assays (12).[3] The other two isozymes, the tissue unspecific (liver-bone-kidney) and the intestinal alkaline phosphatases have so far not been identified as typical oncodevelopmental antigens. One major reason for this is that the high normal circulating serum levels of these isozymes make a minute addition of tumor derived isozyme difficult to trace. However, elevated serum levels of AP in cancer patients are often seen, although no involvement of the liver is observed.

By means of sensitive discriminating assays capable of measuring each of the three major phosphatase isozymes (12) without any cross-reactivity,[3] it has been possible to make several basic observations presented in this report. The first is the identification in the normal testis of all three phosphatase isozymes. So far the appearance of the intestinal form of the enzyme in amounts equal to those of PLAP has not been described earlier, whereas the identification of AP and trace amounts of PLAP has been recognized (5, 9). It is still too early to evaluate the implications of this finding in relation to the physiological role of this enzyme system.

Another major observation is that all the investigated tumors demonstrate highly increased levels of all three isozymes, not only PLAP, which is related to the fetal-placental unit. This indicates that the entire genome coding for alkaline phosphatases is activated in seminomas. The relative increase is the same for AP as it is for PLAP, demonstrating that the increased expression of isozymes typical of the fully differentiated cells is of the same magnitude as that of the fetally related isozymes. This observation may have physiological implications and is an indicator that the catalytic properties of the enzyme might be of importance for the tumor progression.

The same type of simultaneous expression of different phosphatases occurs in the early placenta, which from the sixth to the tenth week after implantation only expresses AP and after the first trimester of pregnancy switches to synthesis of PLAP (17). The functional role of these enzymes is, however, not yet known. In the seminoma tissue as compared with the mature placenta the dominating form of the phosphatases is still the AP. The increased levels of all isozymes in seminoma tissues are however not necessarily correlated to high serum levels of the same enzymes due to differences in clearance rate.

The mechanisms leading to the appearance in the normal testis and in seminomas of the PLAP-like enzyme, an enzyme which is almost indistinguishable from PLAP, are still a matter of controversy. Differences in reactivity with monoclonal antibodies (6, 18) and inhibition patterns with certain inhibitors have been reported (19). Whether a separate gene for the PLAP-like enzyme exists is not yet known. The present investigation adds some important facts to this controversy. The first is that activity *versus* enzyme protein of PLAP, as measured in pregnancy sera, is constant, indicative of a population of circulating PLAP molecules during pregnancy which is rather homogenous. In tumors and tumor sera a significantly higher variation is observed, indicating that the tumor derived enzymes belong to a population of heterogenous molecules. This observation is supported by the fact that the electrophoretic patterns obtained from starch gel electrophoresis demonstrate not only microheterogeneity within each investigated tumor but also significant differences in surface charge between samples, a variation which is not consistent with the normal placental phenotypic classification system. These observations are important when PLAP

and PLAP-like enzymes are used as tumor monitoring agents and different types of assays are used (catalytic, immunocatalytic, and immunochemical). The existence of a population of tumor derived PLAP-like enzymes which are partially heat sensitive may indicate a loss of information when catalytic techniques are used. Decreased catalytic activities in tumor sera after heat denaturation were observed in contrast to constant catalytic activities in pregnancy sera.

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REFERENCES

1. Stigbrand, T., and Fishman, W. H. Human alkaline phosphatases. Prog. Clin. Biol. Res., *166:* 1–361, 1984.
2. Millan, J. L. Molecular cloning and sequence analysis of human placental alkaline phosphatase. J. Biol. Chem., *261:* 3112–3115, 1986.
3. Kam, W., Clauser, E., Kim, Y. S., Kan, Y. W., and Rutter, W. J. Cloning sequencing and chromosomal localization of human term placental alkaline phosphatase cDNA. Proc. Natl. Acad. Sci. USA, *82:* 8715–8719, 1985.
4. Fishman, W. H., Inglis, N. R., Green, S., Anstiss, L. L., Ghosh, N. K., Reif, A. E., Rustigan, R., Krant, M. J., and Stolbach, C. C. Immunology and biochemistry of Regan isoenzyme of alkaline phosphatase in human cancer. Nature (Lond.), *219:* 697–699, 1968.
5. Wahren, B., Holmgren, P.-Å., and Stigbrand, T. Placental alkaline phosphatase, alphafoeto protein and carcinoembryonic antigen in testicular tumours. Tissue typing by means of cytologic smears. Int. J. Cancer, *24:* 749–753, 1979.
6. Jeppson, A., Wahren, B., Brehmer-Andersson, E., Silfverswärd, C., Stigbrand, T., and Millan, J. L. Eutopic expression of placental-like alkaline phosphatase in testicular tumours. Int. J. Cancer, *34:* 757–761, 1984.
7. Van de Voorde, A., De Groote, G., De Waele, P., De Broe, M. E., Pollet, D., De Boeres, J., Van de Kerchhove, D., and Fiers, W. Screening of sera and tumour extracts of cancer patients using a monoclonal antibody directed against human placental alkaline phosphatase. Eur. J. Cancer Clin. Oncol., *21:* 65–71, 1985.
8. Millan, J. L., and Stigbrand, T. "Sandwich" enzyme immunoassay for placental alkaline phosphatase. Clin. Chem., *27:* 2014–2017, 1981.
9. Chang, C. H., Angellis, D., and Fishman, W. H. Presence of the rare D-variant heat-stable, placental-type alkaline phosphatase in normal human testis. Cancer Res., *40:* 1506–1510, 1980.
10. International Union against Cancer Report: Workshop on Immunodiagnosis. Cancer Res., *46:* 3744–3746, 1986.
11. Köhler, G., and Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (Lond.), *256:* 495–497, 1975.
12. Hirano, K., Iizumi, Y., Hayashi, Y., Tanaka, T., Sigiura, M., Hayashi, K., Lu, Z.-D., and Iino, S. A highly sensitive assay method for human placental alkaline phosphatase involving a monoclonal antibody bound to a paper disc. Anal. Biochem., *154:* 624–631, 1986.
13. Stigbrand, T., Holmgren, P-Å., Jeppson, A., Damber, M-G., and von Schoultz, B. On the value of placental alkaline phosphatase as a marker for gynecological malignancy. Acta Obstet. Gynecol. Scand., *64:* 99–103, 1985.
14. Holmgren, P-Å., and Stigbrand, T. Purification and partial characterization of two genetic variants of placental alkaline phosphatase. Biochem. Genet., *14:* 777–789, 1976.
15. Beckman, L., Björling, G., and Christodoulou, G. Pregnancy enzymes and placental polymorphism. 1. Alkaline phosphatase. Acta Genet., *16:* 59–73, 1966.
16. Fishman, W. H., Inglis, N. R., Green, S., Anstiss, C. L., Gosh, N. K., Reif, A. E., Rustigan, R., Krant, M. J., and Stolbach, L. L. Immunology and biochemistry of Regan isoenzyme of alkaline phosphatase in human cancer. Nature, *219:* 697–699, 1968.
17. Fishman, L., Migayama, H., Driscoll, S. G., and Fishman, W. H. Developmental phase-specific alkaline phosphatase isoenzyme of human placenta and their occurrence in human cancer. Cancer Res., *36:* 2268–2273, 1976.
18. Wahren, B., Hinkula, J., Stigbrand, T., Jeppsson, A., Andersson, L., Esposti, P. L., Edsmyr, F., and Millan, J. L. Phenotypes of placental-type alkaline phosphatase in seminoma sera as defined by monoclonal antibodies. Int. J. Cancer, *37:* 595–600, 1986.
19. Millan, J. L. Placental and placental-like alkaline phosphatases. Umeå University Medical Dissertations. New Series No. 107. Department of Physiological Chemistry, University of Umeå, Umeå, Sweden, 1983.

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