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ARTICLE

Alkaline Phosphatase Histochemistry and Biochemistry in the Diagnosis of Complete Hydatidiform Mole

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The purpose of this study was a complementary method to the diagnosis and prognosis of complete hydatidiform mole (CHM) and differentiate it from the other cases of gestational trophoblatic diseases. This was done by examining the quality and quantity of the total and the placental alkaline phosphatase activity. The ALP in the tissues and sera from 12 patients were compared with 13 control normal non-pregnant and 30 control pregnant females. The enzyme activities were determined by biochemical and histochemical examination.The placental tissues were obtained from uterine curettage, or after delivery which then were frozen in a liquid nitrogen and processed for biochemical study. Cryosections were histochemically stained for ALP and PLAP by the azo coupling method. Isoenzyme specificity was evaluated by heating the tissue at 65°C for 15 min while the including L-phenylalanine (50 mM), D-phenylalanine (50 mM) and L-homoarginine (50 mM) were used for chemical inhibition study. The activity of ALP and PLAP of patients were reduced in comparison with pregnant control group ($P<0.05$). There was no significant difference between the patients and non-pregnant control ($P<0.05$) group. The localization of enzyme activities in cryosections of all groups were in the basal, apical, and the cytoplasm of syncytiotrophoblast cells. The ALP in all the groups was thermostable (65°C for 15 min) and was inhibited by L-phenylalanine, but no inhibition was seen with L – homoarginine in patients group only. These findings suggest that the PLAP is a useful marker in the diagnosis and prognosis of hydatidiform mole. (Pathology Oncology Research Vol 6, No 2, 105–110, 2000)

Keywords: alkaline phosphatase; placental alkaline phosphatase; complete hydatidiform mole; gestational trophoblastic disease

Introduction

Gestational trophoblastic disease (GTD) is a proliferation of trophoblastic cells and in general this term includes hydatidiform mole, invasive mole, placental site trophoblastic tumor and choriocarcinoma. The hydatidiform mole (HM) is a benign condition that may be seen in two forms, complete or classic form (CHM) and partial hydatidiform mole which can transform to a malignant tissue.[14,26] The incidence of HM has been studied extensively by any investigators.[3,13] The frequency of HM in Iran was reported to be 3.18 per thousand pregnancies.[2] Since GTD is derived from trophoblastic cells that secret human chorionic gonadotropin (hCG), the measurement of urinary and serum hCG have been used to diagnose and elauate the response of such neoplasia to chemotherapy.[22,27] However in some cases a misleading result can be obtained, for example, the serum a patient who has cancer and then becomes pregnant, can show a high level of hCG. On the other hand, a high level of this marker can be elevated without any background of of CHM.[7,9,24]

Alkaline phosphatase was used as a reliable marker for several types neoplasia.[19] The human placental type

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Abbreviations & Units: HM: Hydatidiform mole; CHM: complete hydatidiform mole; GTD: Gestational trophoblastic disease; ALP: Alkaline phosphatase; PLAP: Placental alkaline phosphatase; L-ph: L – phenylalanine; D – ph: D -phenylalanine; L – homo: L – homoarginine; hCG: human chorionic gonadotropin; M: mol; mM: milli mol; u/mg: unit per milligram; mg: milligram; H & E: Hematoxylin and Eosin stain.

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Table 1. **Specific activity of total ALP in serum and tissue extract**

Sample	*Number*		*Total protein (m)*	*Total activity (u)*	*Specific activity (u/mg)*
Serum	13	$\overline{X}$ ± SD	6.55 ± 0.14	217 ± 87.28	0.00327 ± 0.0011467
Non-pregnant female		Range	6.5 – 7	153 – 485	0.00173 – 0.00746
Pregnant female	30	$\overline{X}$ ± SD	6.55 ±1.59	657.3 ± 249.74	0.0101 ± 0.00391
		Range	6.1 – 7.6	279 – 1355	0.0042 – 0.022
Hydatidiform	10	$\overline{X}$ ± SD	6.82 ± 0.63	314.2 ± 119.08	0.00435 ± 0.00225
Mole patient		Range	6.1 – 7.8	150 – 466	0.0001 – 0.0076
Tissue extract	11	$\overline{X}$ ± SD	2.93 ± 0.64	13420 ± 5561.93	0.455 ± 0.1458
Pregnant female		Range	1.9 – 3.7	7370 – 26040	0.269 – 0.786
Hydatidiform	12	$\overline{X}$ ± SD	3.52 ± 1.470	810.83 ± 571.5	0.0303 ± 0.0242
Mole patient		Range	1 – 6.2	150 – 2260	0.003 – 0.090

Abbrevation: $\overline{X}$: mean; SD: standard diviation; u/mg: unite per milligram.

alkaline phosphatase is a major cell surface sialoglycoprotein of placental syncytiotrophoblast plasma membranes.[23] This membrane forms an extensive interface between fetal tissue and maternal blood. As the gestation progresses the concentration of PLAP increases and this can be cauesed by the detachment of ALP from the membrane into the maternal circulation.[20] This enzyme is characterized by its ability to hydrolyse phosphoester bonds at alkaline pH. This is coded by a separate gene locus which is different from other human isoenzymic forms of ALP.[23] The full term PLAP is identified biochemically by its extreme resistance to denaturation by heat (65°C).[6] This enzyme can be inhibited by L-ph (75%) and resists inhibition by L-homo.[5,17] It can also be detected in tissues by immunohistochemical methods.[8,10,11,12,18]

Bur was the first investigator to report the histochemical reaction of ALP in HM, invasive mole and choriocarcinoma.[4] Many attempts were made to predict the prognosis or the recurrence of a mole in pregnant patients as well as the risk of the development of malignancy. Beckstead also showed that all syncytiotrophoblast cells had reaction for ALP.[1] Immunohistochemical methods confirmed the above results.[10,11,18] Losch and Kainz[18] and Danihel et al reported that the syncytiotrophoblast cells of CHM and choriocarcinoma had weak expression of PLAP while the expression of PLAP in partial hydatidiform mole was strong.[10,11]

Our study has focused on the measurment of activity of ALP and PLAP in the serum and tissues of CHM patients by biochemical and histochemical methods, to diagnoses or evaluate the prognosis of CHM.

Table 2. **Specific activity of PLAP in serum and tissue extract**

Sample	*Number*		*Total protein (m)*	*Total activity (u)*	*Specific activity (u/mg)*
Serum	13	$\overline{X}$ ± SD	6.55 ± 0.14	54.69 ± 31.23	0.00066 ± 0.00050
Non-pregnant female		Range	6.5 – 7	3 – 86	0.00004 – 0.00173
Pregnant female	30	$\overline{X}$ ± SD	6.55 ± 1.59	229.2 ± 155.31	0.00456 ± 0.00226
		Range	6.1 – 7.6	143 – 747	0.00228 – 0.0109
Hydatidiform	10	$\overline{X}$ ± SD	6.82 ± 0.63	3.5 ± 3.27	0.00005 ± 0.00004
Mole patient		Range	6.1 – 7.8	0 – 8	0 – 0.001
Tissue extract	11	$\overline{X}$ ± SD	2.93 ± 0.64	13154.5 ± 6952.1	0.430 ± 0.18744
Pregnant female		Range	1.9 – 3.7	3500 – 27580	0.166 – 0.835
Hydatidiform	12	$\overline{X}$ ± SD	3.52 ± 1.470	117.5 ± 110.9	0.00439 ± 0.00414
Mole patient		Range	1 – 6.2	0 – 280	0 – 0.013

Abbrevation: $\overline{X}$: mean; SD: standard diviation; u/mg: unite per milligram.

Materials and Methods

The serum samples were obtained from 13 non – pregnant, 30 pregnant and 10 hydatidiform mole females. All samples were stored in –20°C until the assay was carried out. Trophoblastic tissues from 12 complete hydatidiform moles were obtained after dilatation and curettage and stored in liquid nitrogen until histochemical and biochemical studies were performed. Thirty full term control normal placentas were obtained just after delivery and were frozen in liquid nitrogen as well.

Tissue sections 3–4 micrometer thick were cryosectioned at –30°C and used as fast as possible. Tissue extraction was carried out as follows: tissue fragments weighing about 3 g were homogenized in 3 volumes of 0.01 M Tris-HCl buffered saline (pH=8.3) per gram of tissue. After filtration and centrifugation at 7000 g for 15 min the supernatants were used as tissue extract.

The alkaline phosphatase assay was carried out by using 10 mM *P* – nitrophenyl phosphate as a substrate in 1 m/L diethanolamine buffer (pH=9.8) containing 0.5 mM $MgCl_2$. *P*-nitrophenol released by enzymatic activity which gives the solution a yellow colour at alkaline pH with an optical density of 405 nm. One unit of ALP activity (u) is expressed as 1 mM of *P*-nitrophenol released per min and the specific activity is expressed as enzyme activity per milligram of protein in solution. Protein concentration was determined by the Biuret method.[25] Serum and tissue extracts were heat-inactivated by placing them in stoppered test tubes containing 2 ml of samples in a water bath at 65°C for 15 min. The water level was at least 3 cm above the samples.

The enzyme histochemistry for ALP was carried out by an azocoupling technique.[1] The heat stability of ALP was determined by the Koid method[16] which was modified by putting the sections in an incubator for 15 min at 65°C before the incubation in the substrate medium containing the naphthyl AS-B1 phosphate (Sigma Chemical CO.Ltd, England). For chemical inhibition, sections were preincubated for 1 hour with the inhibitors (50 mM L-homo, 50 mM D-ph or 50 mM L-ph) and the same concentration of these inhibitors were added to the incubation medium which contained the substrate at 4°C.[1] Hematoxylin and eosin were used as routine staining.

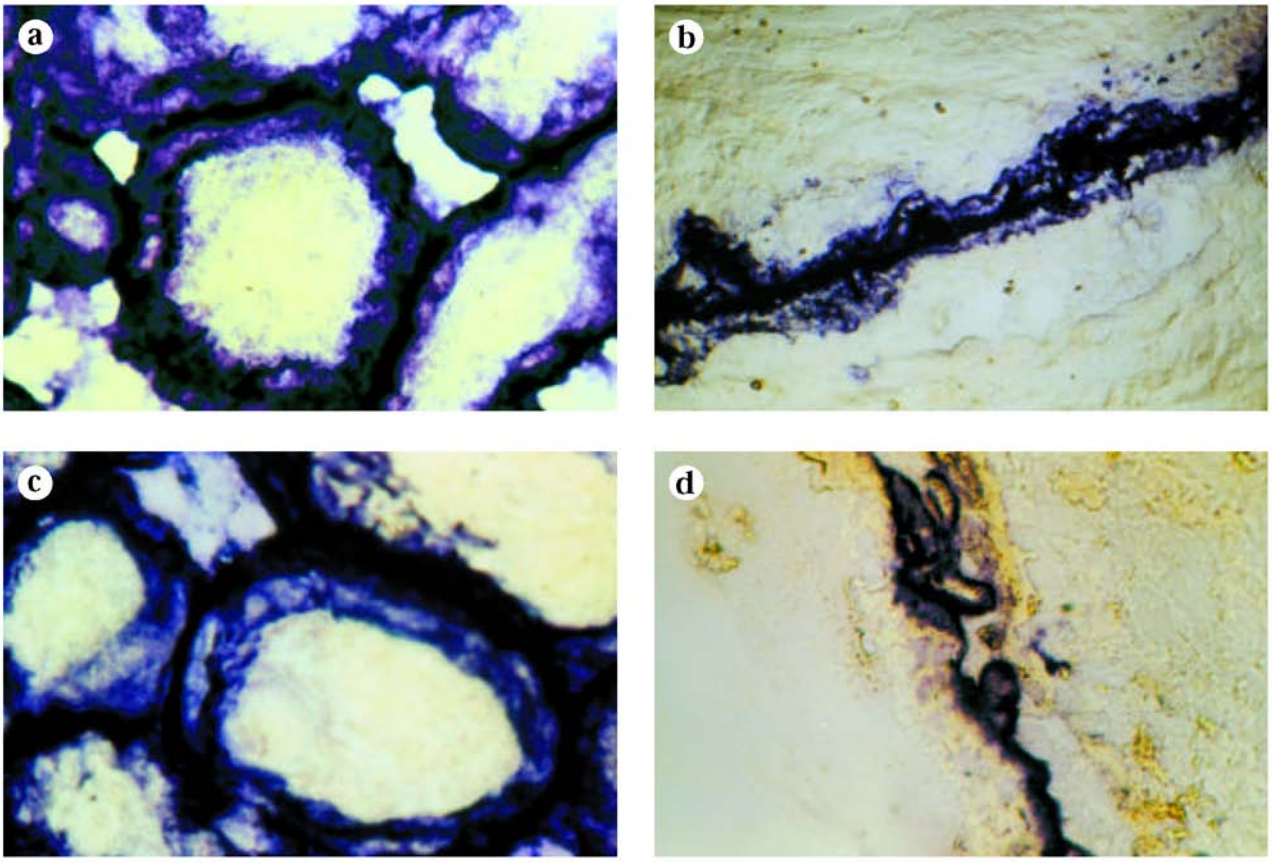

Figure 1. a) *The alkaline phosphatase activity in term placental villi without any treatment neutral red as counter stain × 720.* ***b)*** *The AIP activity in CHM villi × 216. Note the activity of AIP observed in apical, basal and cytoplasm of syncytiotrophoblast cells of term placcenta and CHM.* ***c)*** *PLAP activity of term placental villi after heat inactivation (65c for 15 min) × 720* ***d)*** *PLAP activity of CHM villi after heat inactivation × 360.*

Statistics

An unpaired student's t-test was used to determine the significant differences between enzyme activity in patients, non-pregnant control and pregnant controls. In all cases a P value <0.05 was selected as the minimal criterion for statistically significant differences.

Results

Biochemical findings

The total ALP and PLAP activity in the serum and tissue extracts of CHM were decreased in comparsion to the normal pregnant females (P<0.05) (***Table 1, 2***). There was no significant difference between the non pregnant females and the patients (P<0.05) These results show severe hypophosphatasia in serum and tissue extracts of CHM patients.

The normal non-pregnant females had some heat stable alkaline phosphatase activity (range: 0.0004 u/m – 0.00173 u/m). This may be due to the 1–2% of the non-placental ALP isoenzymes which remain uninhibited by heat (65°C), or to artifact.

Histochemical findings

At the light microscopic level all villi of normal placenta were strongly positive for ALP and PLAP. The pattern of enzyme distribution was regular, the reaction could be observed and the apical membrane (brush border) and basal membrane of syncytiotrophoblast cells (***Figure 1a***), while the cytoplasm of these showed granular distribution of the enzyme. In contrast to full term placenta, the CHM villi showed irregular distribution of enzyme activity (***Figure 1b***) and many villi did not show any reaction. The location of the enzyme activity was determined in the apical and basal synsytiotrophoblast cells border of the positive villi. In both groups ALP was heat stable at 65°C for 15 min (***Figures 1c,d***). However, L-ph was a strong inhibitor for ALP in cells from full term placenta (***Figure 2a***), whereas less inhibitory effect was observed in CHM cells with this inhibitor (***Figure 2b***). D-ph and L-homo were not effective inhibitors for both groups (***Figures 2c,d***). ***Table 3*** shows the ALP isoenzyme specificity of CHM and full term placenta which were used as control on a five grade scale of 0 to 4 in the study. This grading system was used according to Beckstead.[1]

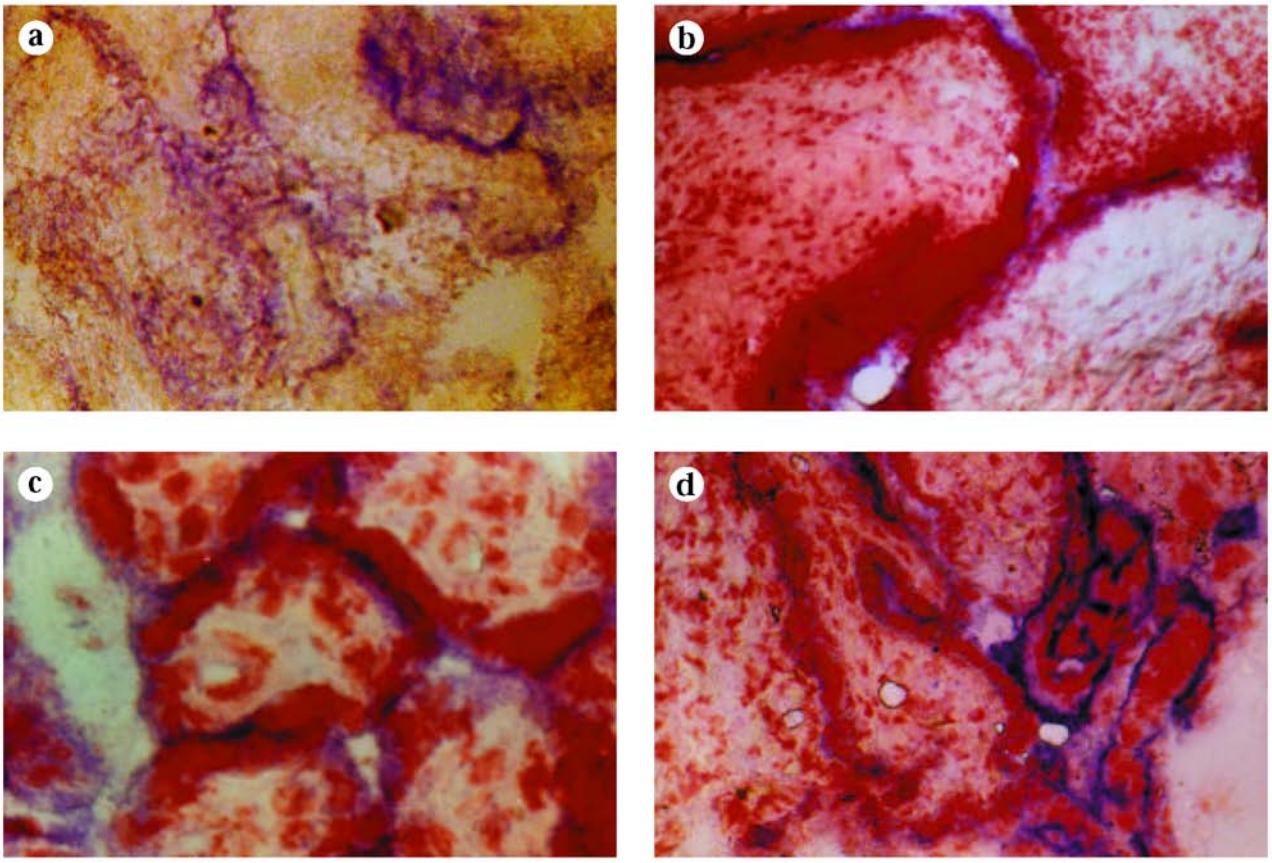

Figure 2. a) *Alkaline phosphatase activity after treatment with 50 mM L-ph in term placental villi × 360.* ***b)*** *50 mM L-ph in CHM villi, neutral red as counter stain × 216.* ***c)*** *50 mM L-homo in term placental villi, neutral red as conter stain × 720.* ***d)*** *50 mM L-homo in CHM villi, neutral red as conter stain × 360.*

Table 3. **Alkaline phosphatase isoenzyme specificity**

Tissue	*Control* [(a)]	*L – ph (50 mM)*		*D – ph (50 mM)*		*L – homo (50 mM)*		
		max	*min*	*max*	*min*	*max*	*min*	*heat stability*
Term placenta	++++	+	+	+++	+	+++	++	++++
percent (%)	100	66.7	14.8	36	16	29.2	37.5	100
Hydatidiform mole	++++	++++	++	++++	++	+++	+	++++
percent (%)	(b)	56.6	56.6	11.1	45.5	23.2	11.1	100

(a) All controls rated as ++++ for evaluation of inhibition
(b) If villi of hydatidiform mole had ALP activity we could see the results,
(min) minimum ALP activity observed,
(Max) maximum ALP activity observed.

Discussion

The biochemistry of serum as well as tissue extract, and the histochemical findings in CHM patients showed that the activity of total ALP and PLAP were decreased in comparison with the control pregnant group. These observations have also been made by other investigators.[1,4,11,18] Some of them showed weak expression of PLAP by syncytiotrophoblast cells of CHM by immunohistochemical methods.[11,18] The CHM hypophosphatasia may be due to high grade of neoplasia, and may be caused by the suppression of many proteins' and enzymes' synthesis including ALP. Another explanation is that posttranscriptional ALP modification may be defective resulted in a change in enzyme activity.

The localization of enzyme in histochemical cryosections obtained from molar tissues was irregular. In contrast to this, the full term placenta had a regular reaction. The explanation of the above results may be the irregular proliferation of syncytiotrophoblast cells in molar villi. The variable pattern of the enzyme activity in CHM was noticed mainly at the brush border of the apical portion of the syncytiotrophoblast cells, where the enzyme is located.[23] The characteristics of ALP in CHM cells were different from that of the normal fullterm placental cells. A low sensivity to L-ph, moderately sensitivity to L-homo and the heat resistance have been documented by others.[5,6,17] An explanation for the differences between the activity of ALP in patients and normal controls may result from hybridization between two different genes of PLAP, it was reported that normally one isoenzyme gene is expressed at an early stage of trophoblast development and then turned off at approximatly 12 weeks, while the other isoenzyme gene is not expressed until 10 weeks of gestation and continues to be active during the rest of the period of gestation, and that this pattern may be changed in malignancy.[21]

ALP is probably an important component in the transport of sugar and phosphate across the trophoblast cell membrane,[15] and the reduction of ALP may be the cause of the hydropic nature of hydatidiform mole in placental villi.

Routine serological and biochemical methods for the diagnosis of hydatidiform mole are based on the measurment of β-subunit of human chorionic gonadotropin in patients' serum.[22,27] The drawback of these methods is the high rate of false positive results, which may be due to presence of pregnancy or a paraneoplastic feature of a remote tumor which can produce hCG. Thus measurement of ALP and PLAP activity in serum and molar tissue extract could be a complementary method for the diagnosis or monitoring of CHM.

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