Alkaline Phosphatase of Cancerous Larynx Tissue in Comparison with the Placental Enzyme

Biochemical and Histochemical Studies

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Tissue specimens of squamous cell carcinoma of the larynx from twenty patients were processed for histological and histopathological characterization. A histochemical study of alkaline phosphatase (ALP) was carried out using the simultaneous azo coupling method, and biochemical studies were performed using disodium phenylphosphate as substrate. Full-term, normal placentae were used for comparison. The specific activity of ALP from cancerous laryngeal tissue was 8.9 mKAU/mg protein compared with 154.7 mKAU/mg protein in the placenta. The ALP was localized histochemically in tumor cells (tumor-specific), blood vessels (vascular) and fibrous tissue (interstitial). The tumor-specific phosphatase was sensitive to inhibition by L-phenylalanine, L-leucine and to a lesser degree by L-tryptophan and levamisole. Placental ALP, on the other hand, was completely inhibited by levamisole, more resistant to leucine and more sensitive to phenylalanine and tryptophan. Biochemical estimation of ALP in cancerous laryngeal tissue combined with inhibition studies revealed that the tumor-specific activity of ALP constitutes 15% of the total ALP activity while the major isoenzyme was the vascular ALP, and around one-third of ALP activity was attributed to the interstitial enzyme. The characterization and localization of these isoenzymes are described and compared with that of the placenta. The significance and implications of the above findings are presented.

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Alkaline phosphatase (ALP) is a ubiquitous membrane-bound ecto-enzyme. Different isoforms of the enzyme are identified; among these are the intestinal, placental or the placental-like as well as the tissue unspecific: liver, kidney and bone (LKB) isoenzymes. Placental ALP (PLAP) is usually synthesized in the placenta and becomes detectable in the maternal circulation after the twelfth week of pregnancy (1). The most remarkable property of PLAP is its pronounced heat-stability, which provides a convenient and specific test for the presence of this isoenzyme. The carcinoplacental isoenzymes are regarded as phase-specific oncofetal gene products (2) that share antigenic determinants with normal placental ALP (3) and have proved to be useful in diagnosis and in predicting prognosis (4, 9). Most of these isoenzymes are remarkably stable to heat (6, 7). Currently, Regan, Nagao and Kasahara isoenzymes are considered as biomarkers of malignant tumors. The Regan isoenzyme is a heat-stable glycoprotein present in the placenta and cell membranes of different human malignant neoplasms and/or sera of patients suffering from tumors (8). It is highly sensitive to L-phenylalanine (Phe). The Nagao isoenzyme is inhibited by L-phe, L-leucine (Leu), L-isoleucine (ILE), L-valine and EDTA.

Biopsy material from tumor larynx tissue revealed an elevated heat-stable alkaline phosphatase (HSALP) (9). This HSALP was also detected in patient's sera, declining with good prognosis and increasing with tumor progression in squamous cell carcinoma of the larynx (5). Histochemically, it was resistant to Phe inhibition and was localized in tumor cells.

The aim of this study was to investigate ALP in squamous cell carcinoma of the larynx. This included the histopathological and histochemical study of tissue alkaline phosphatase in total laryngectomy specimens. The inhibitor profile was considered in an attempt to obtain preliminary kinetic parameters of the various ALP isoenzymes. Since most ALP isoenzymes linked to tumor biology are heat-stable, it was deemed prudent to perform a comparison with the well-known and defined heat-stable

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PLAP. No other report has so far been published concerning ALP in cancerous larynx tissue with the exception of a short communication by Hammond et al. (10), who noted a slight elevation of the total activity of ALP in eight biopsy specimens from cancerous larynx tissue.

MATERIAL AND METHODS

Whole larynx with the hyoid bone and parts of the strap muscles of the neck were obtained after total laryngectomy from 20 patients aged 35–65 years with a mode of 60 years. Each patient undergoing this operation had a preoperative biopsy with a histopathological diagnosis of squamous cell carcinoma. The larynx was transferred in dry ice bags to the laboratory within 30 min. The larynx was opened from behind and the two sides were pushed back to expose the interior of the larynx. Identification and demarcation of the tumor was carried out, some being identified as supraglottic, others as transglottic and some as ulcerative tumors invading the upper part of the thyroid cartilage.

The specimens were cut into small pieces each 0.1–0.5 g and stored in liquid nitrogen at −176°C.

Full-term placentae were collected from normal vaginal deliveries of mothers who had a normal uneventful pregnancy, giving birth at the fortieth gestational week.

ALP histochemistry

The fresh chopped tissue pieces (larynx and placenta) were rapidly frozen by drenching in liquid nitrogen, then mounted on cryostat chunks and sectioned by Frigocut-N 2800 cryostat. Fresh, frozen sections of 10 μ thickness were obtained and stored at −20°C. Specimens were processed for histological and histopathological examination. The simultaneous azo dye coupling method for histochemical detection of ALP was used (11).

ALP biochemistry

The biochemical method of Kind & King (12) was used to detect the activity of ALP in tissue extracts. The results were expressed in KAU/dl using disodium phenylphosphate as substrate.

Heat inactivation was done on tissue extracts at 65°C for 15 min and on tissue sections at 65°C for 30 min. The chemical inhibitors used were: levamisole, Phe, Trp and Leu.

Parts of the cancerous and placental sections were chopped into pieces, crushed in a mortar and resuspended in 3 volumes of homogenizing buffer (10 mM/l Tris-HCl, pH7.5, 0.15 mol/l NaCl, 3 mmol/l phenyl methyl sulphonyl fluoride, 1 mmol/l 2-mercaptoethanol and 0.5% glycerol) (13). Homogenization was done in an ice jacket using a kinematical homogenizer (Karl Kolb, Scientific Technical Supplies) at setting 4 for 5 min. The samples were then sonically disrupted in an ice jacket, using a small probe of a Microson Ultrasonic Cell Distributor at 18 mA for 30 s. Tissue extracts were obtained by centrifugation at 10 000 g for 15 min at +4°C using a Beckman Model J2-21 Refrigerated Superspeed Centrifuge. The supernatant containing the soluble ALP was kept at −20°C for no more than one week.

RESULTS

Histological characterization of processed tissue

Biopsy material obtained from total laryngectomy specimens produced a histopathological picture of malignant invasive squamous cell carcinoma. The samples included full thickness mucous membrane comprising a covering epithelium and an underlying lamina propria. The mucous membrane of the interior of the larynx was metaplastic, i.e. transformed from pseudostratified ciliated epithelium into stratified squamous epithelium. Vascular profiles were observed at the junction of the mucous membrane and the lamina propria forming a subepithelial vascular network. Dysplastic epithelium was also detected. It is evident that the designation 'normal' laryngeal mucous membrane is misleading. This tissue is more appropriately termed 'tumor-associated laryngeal tissue' (TALT). No histological evidence suggesting ossification or calcification was detected.

ALP histochemistry

The final reaction product, 'FRP' of ALP, is a well-localized brownish black deposit on sites of enzymatic activity (Fig. 1). Histochemical activity of ALP was detected in tumor cells. It was found that the intensity of the activity is more pronounced in the lower (deep) zones of the malignant surface epithelium, especially in the prongs of malignant tissue infiltrating the sub-epithelial regions. In transversely cut malignant extensions, the activity of ALP was strong in the peripheral cellular layer in comparison with the deeper region. The histochemical activity is very evident in blood vessels. The third histochemical localization is seen in the interstitial connective tissue between tumor masses and blood vessels laid over the bundles of connective tissue.

Samples of TALT mucous membrane, tested for ALP activity, failed to show any histochemically detectable activity in the epithelial component of the mucous membrane. Full-term placental samples showed strong ALP activity. The main site of localization was in the chorionic trophoblastic layer. Some of the fetal blood vessels showed activity of the enzyme, others not at all.

Histochemical inhibition profile of ALP

Placental samples treated with heat, showed the persistence of ALP histochemical activity at the trophoblastic part of the chorionic villi (Table 1).

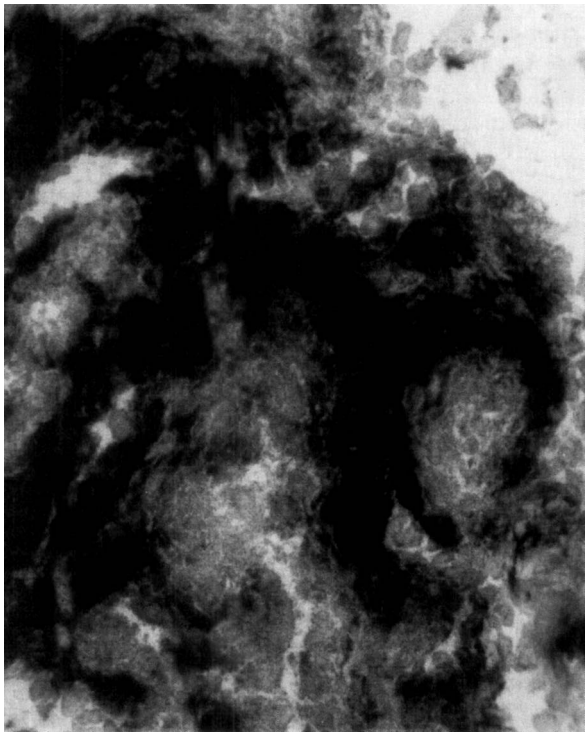

Figure. Alkaline phosphatase histochemistry. Laryngeal squamous cell carcinoma specimen. Intense reactivity is localized in tumor masses (black). Fresh frozen section.

It was not possible to obtain heat inactivation of ALP in tumor sections. The tissue was friable and did not adhere to the coverslips despite gelatinization. Most sections were lost in the pre-incubation bath which halted reproducible and consistent processing of the tissue. The degree of inhibition was measured subjectively, + + + denoting complete, + + partial + slight and 0 no inhibition.

L-phenylalanine (10 mM), profoundly inhibited ALP of tumor masses (+ + +) that were found in both the surface malignant epithelium and the penetrating invasive prongs (Table 1). ALP in blood vessels and ALP in the interstitial connective tissue were not affected by this inhibitor.

Tryptophan (0.5 mM) also inhibited the enzyme in tumor mass, but there was a residual activity which escaped inhibition and thus the degree of inhibition was graded (+). The same observation was noted for the interstitial tissue enzyme, whereas that of blood vessels was not affected at all by this inhibitor.

Levamisole pre-incubation (5 mM), abolished all the enzymatic activity in blood vessels and that of the interstitial tissue to a lesser degree. The activity of ALP in tumor cells was preserved to some extent.

L-leucine (10 mM), had a strong inhibitory effect on ALP in tumor cells and blood vessels, leaving only the diffused interstitial activity of ALP to be demonstrated.

The placental sections run along with the tumor specimens showed a different profile of inhibition. They showed the same sensitivity to Phe but were not affected by Trp.

Leu had no effect on the demonstration of ALP in the chorionic villi, while levamisole produced complete inhibition. The startling observation was that ALP activity of tumor cells was very sensitive to Leu, whereas placentral ALP was resistant (Table 1).

Biochemical activity of ALP

After heating the cancerous laryngeal tissue extracts to 65°C for 15 min, the percentage of HSALP activity ranged from 1.4 to 17.7% of the total ALP activity with a mean + SD of 7.7 + 6.1%. Converted to specific activity, the range was 0.19–2.14 mKAU/mg protein with a mean + SD of 0.63 + 0.52 mKAU/mg protein (Table 2).

Levamisole produced the greatest inhibition of total ALP activity in cancerous larynx tissue ranging from 50.3 to 99% with a mean + SD of 86.9 + 13.3%. Leu inhibition ranged from 50.2 to 88.9% with a mean + SD of 69.0 + 10.4% (Table 2).

The two aromatic amino acids Phe and Trp produced a mean + SD of 11.9% and 13.0% inhibition respectively with a range for Phe between 1.3 and 71.3% and that for Trp between 1.7 and 60.5% (Table 3).

A strong positive correlation was found between the percentage inhibition by Phe and the percentage of heat-stable ALP activity (r = 0.85). In view of the histochemical evidence for the tumor cell ALP inhibition by Phe, this result indicates that the tumor cell enzyme is heat-resistant. The weaker correlation (r = 0.28) between the percentage

Table 1

Chemical inhibitor profile of alkaline phosphatase histochemical activity in the chorionic villi, tumor cells, blood vessels and interstitial tissue

Inhibitor	Conc. used	Placenta Chorionic villi	Tumor tissue		
			Tumor cells	Blood vessels	Interstitial tissue
L-phenyl alanine	10 mM	▦	□	■	■
L-leucine	10 mM	■	□	□	■
L-tryptophan	0.5 mM	▦	▨	■	▨
Levamisole	5 mM	□	▨	□	▦

Degree of inhibition: (Complete inhibition) (+ + +) = □; (+ +) = ▦; (+) = ▨; (−) = ●; (No demonstrable inhibition).

Table 2

Total alkaline phosphatase activity (ALP) and the heat-stable fraction (HSALP) in laryngeal tumor tissue and placenta

Tumor samples	ALP KAU/g tissue	Specific activity of total ALP mKAU/mg protein	HSALP %	Specific activity of HSALP mKAU/mg protein
1	0.55	8.2	3.7	0.30
2	0.56	13.6	1.4	0.19
3	1.06	16.9	2.3	0.38
4	0.29	8.8	2.1	0.19
5	0.49	4.4	8.6	0.37
6	0.82	6.2	6.5	0.40
7	0.71	9.5	5.3	0.50
8	0.56	12.3	17.5	2.14
9	0.54	4.9	17.7	0.85
10	0.43	7.5	13.3	0.99
11	0.68	5.5	14.2	0.78
12	0.98	13.1	0.2	0.73
13	0.50	4.9	7.8	0.38
Mean	0.63	8.9	7.7	0.63
S.D.	0.22	3.9	6.1	0.52
n	13	13	13	13
Placenta mean	5.52	154.7	96.35	148.85

inhibition by Trp and the %HSALP is to be expected as this inhibitor also affects the interstitial fibrous tissue isoenzyme.

The mean value of the total ALP activity of three placentae specimens was 5.52 mKAU/g. The activity was ninefold greater than that of the tumor (0.63 mKAU/g) and was predominantly heat-resistant (96.35%) (Table 2). The inhibition rates of the placental ALP by Phe, Leu, Trp and levamisole were 21.6%, 32.0%, 55.9% and 63.6% respectively. The processed placental samples showed a uniform activity of ALP.

DISCUSSION

The total ALP activity of cancerous laryngeal tissue was initially determined in extracts of 0.1–0.5 g pieces. Variability in the results with different specimens of the larynx necessitated treatment of the cancerous tissue of a single larynx as a pool. This above-mentioned variability is explained histochemically on the basis of different contributions to the pool, of tumor cell, interstitial fibrous tissue and vascular ALP components. Hammond et al. (10) claimed that ALP activity was increased in cancerous laryngeal tissue and the increase was consistent with histological observation of increased osteoblastic activity and ossification at the site of invasion. However, we did not find such a correlation, either histologically or biochemically.

ALP activity in the placental extracts obtained by the same method was found to be ninefold higher than that of the laryngeal tissue extracts. This placental enzyme was found to be predominantly heat-stable (96.35%), a result in agreement with that reported by Chuang (14) and very close to that reported by Kaplan (15) indicating the predominance of the heat-stable isoenzyme. The PLAP has the capacity to bind the Fc portion of human IgG in the surface membrane of syncytial trophoblasts and is suggested to participate in the transfer of IgG from the maternal circulation to the fetus (16).

The mean percentage of the HSALP activity in cancerous laryngeal tissue was 7.73%. Combined with the evidence of a heat-stable enzyme in serum, this result clearly denotes that this isoenzyme forms a minor component of the total ALP activity detected in cancerous laryngeal tissue. However, it is an important component since it is the source of the heat-stable enzyme in the serum which can be exploited for practical purposes (5). The cancerous laryngeal tissue contains a pool of different isoenzymes of ALP and not a single predominant enzyme as in the case of the placenta.

A histochemical study revealed that ALP is localized in three major sites: tumor cells, blood vessels and interstitial fibrous tissue (TVI isoenzymes).

The Phe inhibitable component is specific to tumor cells as observed histochemically. Biochemical evaluation of such an inhibition suggests that the tumor-specific ALP contributes to about 15% of the total pool. Trp partially inhibited the tumor-specific ALP (TSALP). These two aromatic amino acids have previously been found to inhibit laryngeal tumor ALP and other oncofetal ALPs (13, 14, 17, 18) Leu inhibited ALP of tumor cells and blood vessels but not that of the interstitial fibrous tissue. The difference in the percentage inhibition between that caused by Leu and that affected by Phe suggests that the vascular ALP comprises about 50% of the total pool and thus makes the major contribution to the pool.

Variations in the vascularity between different tissue specimens as well as the relative contribution of tumor cells and interstitial fibrous tissue to the processed tissue greatly affect total ALP estimation.

The remaining one-third of activity (about 35%) includes interstitial tissue ALP and ALP from other sources, for example from serum contained within the tissue mass. This conclusion is supported by the levamisole inhibition profile.

This compound produced the greatest degree of inhibition of ALP activity (86.9%) and seems to be a broad spectrum inhibitor of blood vessels and interstitial tissue ALP; TSALP showed a much lower degree of inhibition. Zoelliner & Hunter (19) reported an inhibition of LKB isoenzymes in vascular tissue by levamisole but no report could be traced on its inhibitory properties in onco-fetal ALP.

Placental ALP and the tumor-specific ALP (TSALP) were found to be profoundly inhibited by Phe. Trp, however, proved to be a less potent inhibitor of TSALP. It was found that Leu profoundly inhibited TSALP but not

Table 3

Biochemical inhibition of ALP of cancerous larynx tissue and placenta

Tumor samples	Percentage inhibition of ALP activity by			
	L-phenylalanine	L-leucine	L-tryptophan	Levamisole
1	1.3	76.7	30.3	50.3
2	71.3*	73	60.5*	75.9
3	1.7	68.9	1.7	86.2
4	2.3	66.1	5.8	94.4
5	16.1	77.2	16.3	85.5
6	1.1	62.6	7.7	96
7	15.1	53.2	15.1	90.5
8	19.2	76.7	3.3	96.9
9	29.6	71.7	21.4	99
10	20.1	62.9	37.1	91.2
11	20.2	68.6	8.8	98
12	8.1	88.9	2	88.9
13	9	50.2	9.1	76.4
Mean	11.9	69.0	13.0	86.9
S.D.	9.4	10.4	11.6	13.3
n	13	13	13	13
Placenta mean	21.6	32.9	55.9	63.6

(*): offshooting value, excluded according to smoothed distribution curve.

PLALP and thus may serve as a differentiating parameter. The resistance of the TSALP to levamisole segregates it from vascular and interstitial isoenzymes.

The profound inhibition of the TSALP by Phe and the strong positive correlation with the %HSALP activity together with the previous finding of an elevated %HSALP activity in the sera of patients with squamous cell carcinoma of the larynx (5) suggests that the TSALP is heat-stable. Phe sensitivity and heat stability are characteristics of the Regan and Nagao isoenzymes (6, 20, 21). Trp, which partially inhibited the TSALP, has been found to inhibit other heat-resistant ALPs (12, 13). Leu, which inhibits the Nagao but not the Regan isoenzyme (23, 24), profoundly inhibited the TSALP. There is a lack of information about the effect of levamisole and Trp on the heat-stable Regan or Nagao isoenzymes.

In conclusion, this work reports the presence of TSALP in squamous cell carcinoma of the larynx. This enzyme shares, properties with the Nagao isoenzyme. Cytochemical and immunocytochemical techniques based on the detection of TSALP may prove to be useful diagnostic aids, particularly in solving equivocal conditions. The release of this TSALP in serum has already been suggested to serve as a biomarker in squamous cell carcinoma of the head and neck (5). The presence of TSALP can be included within the new horizon of therapeutic modality, namely the specific targeting of therapeutic agents towards tumor cells. Such micro-bullets can perhaps be routed towards TSALP.

This work sets up the essential scaffolding for further studies on the purification, characterization and further practical application of TSALP in squamous cell carcinoma of the larynx.

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