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HEME BIOSYNTHESIS IN HUMAN BREAST CANCER—MIMETIC "*IN VITRO*" STUDIES AND SOME HEME ENZYMIC ACTIVITY LEVELS*

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Abstract—1. Porphyrin biosynthesis from δ-aminoevulinic acid (ALA) was investigated using the technique of tissue explant cultures, in both human breast cancer and its original normal tissue.

2. The activity of ALA-dehydratase, porphobilinogenase and uroporphyrinogen decarboxylase was directly determined in both tumor and normal mammary tissues.

3. Porphyrin synthesis capacity of human breast carcinoma was 20-fold enhanced, as compared with normal tissue, at least between the stages of porphobilinogen and coproporphyrinogen formation.

4. The activity of the three enzymes examined was always lower in normal tissue than in tumoral tissue.

5. Present findings show that porphyrin biosynthesis is increased in breast cancer tissue.

INTRODUCTION

Human breast cancer is one of the most frequent type of malignant tumors found in females, leading to the highest rate of mortality as compared with that due to any other tumors affecting this sex. In practice the treatment of choice is surgery followed by radiotherapy, chemotherapy and hormone therapy. More recently, other treatment reported to be rather effective, has been photodynamic therapy (PDT) (Dougherty *et al.*, 1979), consisting in the administration of the so-called hemotoporphyrin derivatives (HpD) or Photofrin II a more purified product already approved by the FDA for its use in humans. PDT is based on the property of certain porphyrins to efficiently accumulate in neoplastic tissue (Rassmussen-Taxdall *et al.*, 1955; Lipson *et al.*, 1961; Gregorie *et al.*, 1968; Dougherty *et al.*, 1978; Gomer and Dougherty, 1979), and the photodynamic properties of these compounds, which would then allow selective killing of the tumorous cells with little damage to the surrounding normal cells.

A good deal of research on this interesting area has been mostly concerned with the tissular distribution of porphyrins after injection (Gomer and Dougherty, 1979; Bugelski *et al.*, 1981) and the subcellular localization of accumulated porphyrins (Kessel, 1981; Sandberg and Romslo, 1981; Jori *et al.*, 1986). However, the reasons for this porphyrins discrimination between normal and tumor cells are not yet known.

As a first approach to the problem, we consider of value to investigate comparatively the functionality of the heme pathway in both neoplastic and the corresponding normal tissue, from human.

From preliminary studies carried out to establish the optimum experimental conditions, it was found that porphyrin synthesis from the precursor δ-aminolevulinic acid (ALA) is significantly enhanced in explant cultures of human breast carcinoma (Navone *et al.*, 1988).

On the other hand, it is worth recalling that alterations in the drugs detoxifying system, particularly with reference to some heme proteins of the so-named phase I, have been described (Denk *et al.*, 1980; Farber, 1984; Stout and Becker, 1986).

These findings allow us to speculate about the existence of deviations from normal in the heme pathway operating in tumor cells.

So far, information available in this field is only partial and most of it comes from studies using experimental models (Stout and Becker, 1986, 1987; Bonkowsky *et al.*, 1973).

We will present here results on our investigation on the biosynthesis of porphyrins by human breast cancer tissues in comparison with the original normal tissue, using the novel technique of tissue explant cultures and on the other hand we will report on the direct measurement of the levels of some of the heme enzymes in the same kind of tissues. From these findings we can ensure that in human breast carcinoma, porphyrin biosynthesis capacity is notoriously increased, at least from the stage of porphobolinogen (PBG) formation up to that of coproporphyrinogen, when compared with its original normal tissue.

MATERIALS AND METHODS

Tissues

Tissues came from the Instituto Nacional de Oncología "Angel H. Roffo". Human breast carcinoma was used. As

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a matched control, normal mammary tissue from the same patient was used. Samples were obtained after surgical therapy from female adult patients (26–66 years old), with the diagnosis of carcinoma and receiving no treatment; they were kept at 0°C and processed within 4 hr after having been obtained.

Histology

Rutinary histological examination was carried out after fixing in 10% formol, paraffin inclusion, cut and then tinction with hematoxiline and eosine. Every tumor was classified according to its histological malignancy following the recommended international classification for tumors (WHO).

Explant tissue cultures

Fractions of 50–60 mg were kept in the designed culture chamber as described by Vázquez *et al.* (1987) and run under the conditions previously established (Navone *et al.*, 1988).

Porphyrins determination

Porphyrins present in the medium were determined fluorometrically by the procedure and using arbitrary units of fluorescence (FRU) as described and defined by Polo *et al.* (1988). Because of possible cellular lysis, results were also normalized taking into account the degree of lysis, and defined a lysis units (LU) as a function of lactate dehydrogenase (LDH) activity in the medium as already reported (Vázquez *et al.*, 1986). Porphyrin accumulation was therefore measured only in the culture medium and calculated as FRU/mg tissue × LU (Buzaleh *et al.*, 1988).

Homogenates

Enzyme activities were measured in homogenates prepared by disrupting the tissue in 0.25 M sucrose at 4°C at the ratios 1:5 (w/v) for ALA-dehydratase (ALA-D) and 1:3 (w/v) for porphobilinogenase (PBGase) and uroporphyrinogen decarboxylase (URO-D), using an Ultra-Turrax (Janke & Kunkel GmbH & Co. KG, Staufen, F.R.G.) homogenizer. The resulting suspension was centrifuged at 10,000 *g* for 15 min and the supernatant employed for enzymic activity measurements.

Assays of enzymic activity

ALA-D: the method of Batlle *et al.* (1967) was followed.
PBGase: it was determined according to Batlle *et al.* (1978).
URO-D: the procedure of Polo *et al.* (1990) was used.
Proteins: the Lowry *et al.* (1951) technique was employed.
Enzyme units: 1 unit of enzyme activity (U) is defined as the amount of enzyme that catalyzes the formation of 1 nmol of product under the standard incubation conditions. The specific activity (SA) being the enzyme units per mg of protein. It should be noted that in the case of porphyrins formed, they are expressed as FRU, so SA will be FRU/mg protein.

RESULTS

Studies on tissue explant cultures

Serial and comparative studies were performed in samples from 7 patients. It was found that in an average porphyrin formation was enhanced *ca* 20 fold in the neoplastic tissue when compared with the original normal tissue (Table 1). Before and after incubation, histology was examined in each tissue; it was observed that the nuclear area was well preserved instead the cytoplasm had lost definition probably due to cellular membrane damage, it must be noted that, as indicated in Materials and Methods, to account and correct for the probable cellular lysis, LDH activity was measured in the maintenance medium (Vázquez *et al.*, 1986).

Table 1. Porphyrin biosynthesis by both neoplastic and its corresponding mammary normal tissue

Patient	Age	Diagnosis	Histological malignancy	Porphyrin synthesis* N†	Porphyrin synthesis* T‡
1	53	dc§	GIII	0.150	1.23
2	66	dc	GIII	0.130	1.15
3	63	dc	GIII	0.004	1.03
4	40	dc	GII	0.134	1.03
5	26	dc	GII	0.001	2.41
6	30	dc	GII	0.120	3.20
7	62	lc¶	GII	0.001	2.00
$\bar{X}$				0.077	1.72
SD				0.070	0.84
$P \ll 0.001$					

*Expressed as FRU/mg tissue × LU (see Materials and Methods).
†Normal breast tissue.
‡Tumoral tissue.
§Ductal carcinoma.
¶Lobulillar carcinoma.

The number of parenquimatous cells in both neoplastic and normal tissue was also quantified by means of a histological evaluation. Thus, it resulted that the density of cellular population in the former tissue in some cases corresponded to 80% of the sample, while in those samples showing prevalence of fibrous reached to only 20%. In normal mammary tissue values were between 10 and 30%. It should also be noted that no correlation whatsoever was found between the grade of histological malignancy and the magnitude of porphyrin biosynthesis enhancement.

Enzymic activity levels

The above findings, although undoubtedly indicating increased capacity for heme synthesis by the neoplastic tissue would only be an indirect measure of the phenomenon; direct quantification of enzyme activity would reflect more definitely and possibly individualize the changes occurred if any. So, ALA-D, PBGase and URO-D were determined in both neoplastic and normal tissue.

ALA-D

It was measured in samples from 5 patients (Table 2). In every pair activity was higher in tumor than in normal cells, activity of the latter was between 6 and 60% of the corresponding matched malignant cells. However, the variability among different samples was so great that signification tests were negatives. Moreover, ALA-D is usually in excess over the other enzymes of the pathway, therefore we will not risk any definite comment about its relative increased activity in tumor cells, as yet.

Table 2. ALA-D activity in tumor and normal mammary tissues

Patient	Age	Diagnosis	Histological malignancy	SA* N	SA* T	SA* N/T
1	50	dc	GIII	1.32	5.49	0.24
2	66	dc	GIII	0.48	8.45	0.06
3	63	dc	GIII	3.46	5.55	0.62
4	38	dc	GII	0.23	1.74	0.13
5	41	dc	GII	8.27	13.80	0.59

*Expressed as nmol of PBG/mg protein (see Materials and Methods). All abbreviations as in Table 1.

Table 3. PBGase activity in tumor and normal mammary tissues

Patient	Age	Diagnosis	Histological malignancy	SA* N	SA* T	SA* N/T
1	63	dc	GIII	3.67	37.00	0.09
2	42	dc	GII	5.30	13.33	0.39
3	50	dc	GII	0.10	6.70	0.01
4	30	dc	GII	2.50	35.00	0.07
$\bar{X}$				2.89	2.89	
SD				2.19	15.26	
$P < 0.05$						

*Expressed as FRU/mg protein (see Materials and Methods).
All abbreviations as in Table 1.

Table 4. URO-D activity in tumor and normal mammary tissues

Patient	Age	Diagnosis	Histological malignancy	SA* N	SA* T	SA* N/T
1	64	dc	GII	0.61	19.80	0.03
2	50	dc	GII	9.60	20.83	0.46
3	26	dc	GII	11.80	33.00	0.35
4	43	dc	GII	10.30	38.30	0.27
$\bar{X}$				8.08	27.58	
SD				5.06	9.12	
$P < 0.01$						

*Expressed as FRU/mg protein (see Materials and Methods).
All abbreviations as in Table 1.

PBGase

Samples from 4 patients were examined (Table 3). Again activity of PBGase in normal tissue was 1–40% in comparison with paired tumor, and in this case the difference was significant, with $P < 0.05$.

URO-D

Activity was measured in samples from 4 patients (Table 4) and it was between 3 and 46% in normal tissue in comparison with paired tumor, with $P < 0.01$.

DISCUSSION

Previous (Navone *et al.*, 1988) and present results demonstrate that porphyrin pathway is enhanced in tumor, at least from PBG to coproporphyrinogen formation. These findings are in agreement with those found in mice spontaneous mammary tumor (see 'Appendix').

It is important to mention that all attempts to measure δ-aminolevulinic acid synthetase (ALA-S) failed. However, we were able to detect ALA-S in the mice spontaneous mammary tumor; although the levels were low, they were of the same order as those measured in the liver of the same animal (see 'Appendix'). Consequently, in view of the similarity between the enzyme pattern observed in human neoplastic tissue and that coming from the animals, we would attribute the failure in detecting ALA-S in human breast tumor to technical problems. It should be taken in consideration both the know lability of ALA-S and the time elapsed between the obtainment of the samples and their processing.

Concerning to results obtained by others, information is so far scarce and sometimes. contradictory.

Rasetti *et al.* (1967) found that ALA-D was significantly reduced in a variety of tumors of the digestive tract and in the same paper these authors reported increased PBG biosynthesis in neoplastic tissue coming from lung and uterus. On the other hand, this enzyme was 3–5 times lower in leukocytes from patients with mielocytic acute leukemia as compared with controls (Takaku and Wada, 1968), while hydroxymethylbilane synthetase (HMB-S), one of the two components of PBGase, was found to be higher in peripheral lymphocytes of patients with lymphoproliferative diseases such as chronic lymphocytic leukemia and lymphoma (Lahav *et al.*, 1987).

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APPENDIX

N. M. Navone, C. F. Polo, A. L. Frisardi and A. M. C. Batlle

Heme Enzyme Pattern in Mouse Mammary Carcinoma

Abstract

1. We carried out a comparative study on the activity levels of ALA-D, PBGase, HMB-S and URO-D in different mouse tissues including liver, brain kidney and mammary tumor.

2. No significant differences were observed in enzyme activity levels in specimens of the same tissue coming from either normal mice or tumor bearing mice.

3. However, tumoral ALA-D activity was significantly different as compared with the hepatic, renal and brain enzyme. PBGase and HMB-S activities were of the same order in tumor, liver and kidney.

4. The ratio between HMB-S and PBGase was nearly 2 and approximately the same in all the tissues.

5. Tumoral URO-D activity was rather the same of that of liver and kidney.

6. Taking into account the above results we conclude that only a partially altered pattern could exist in neoplastic tissue as compared with a typical hemopoietic tissue such as liver.

7. In spite of this, it is interesting to note the high activity levels of PBGase, HMB-S and URO-D, unexpected in cells derived from a glandular tissue.

Introduction

We have demonstrated that, compared to normal mammary tissue, human breast carcinoma showed an enhancement in porphyrin biosynthesis *in vitro* (Navone *et al.*, 1988). Stout and Becker (1986, 1987) have also found some alterations in the heme pathway both in genetically and chemically induced mouse liver tumor as well as in rat liver nodules and carcinomas. Because of these observations we decided to carry out a comparative study on the activity levels of ALA-D, PBGase, HMB-S and URO-D in different mouse tissues, including liver, brain, kidney and mammary tumor.

Materials and methods

Male mice, strain BALB/c (20–25 g), were used. Animals were fed Purina 3 diet and given water *ad libitum*. Spontaneously mouse mammary carcinoma (M5 type) were used. Mice received a 1 mm^3 *inocula* of tumor, injected under the skin overlying the flanks up to the axilla. The animals were sacrificed one month after the implantation. The mice previously heparinized were killed under ether anesthesia by cardiac puncture and bled.

Homogenates of the tissues were prepared as described by Batlle *et al.* (1967) and the supernatant of 10,000 *g* centrifugation for 20 min was used as enzyme source.

ALA-D activity was measured by the method of Batlle *et al.* (1967); PBGase and HMB-S by the method of Batlle *et al.* (1978); URO-D was determined using the method of Polo *et al.* (1990). Enzyme units were defined as the amount of enzyme that catalyzes the formation of 1 nmol of product under the standard incubation conditions. Specific activity was expressed as U/mg protein. Proteins were measured by the method of Lowry *et al.* (1951).

Results and discussion

No significant differences were observed in enzyme activity levels in specimens of the same tissue coming from either normal mice or tumor bearing mice (Fig. A1). However, tumoral ALA-D activity was significantly different as compared with the hepatic, renal and brain enzymes ($P < 0.01$, 0.01 and 0.05 respectively) [Fig. A1(a)]. On the other hand, PBGase activity levels were of the same order in tumor, liver and kidney [Fig. A1(b)]. This was unexpected because it is often found that enzyme activity levels in cells derived from a glandular tissue differs from that measured in cells such as the hepatocytes, where heme plays an important and active role as prosthetic group of a number of proteins involved in detoxifying systems. Moreover, the ratio between PBGase and ALA-D activities was also different to that found in the other tissues examined [Fig. A1(a, b)].

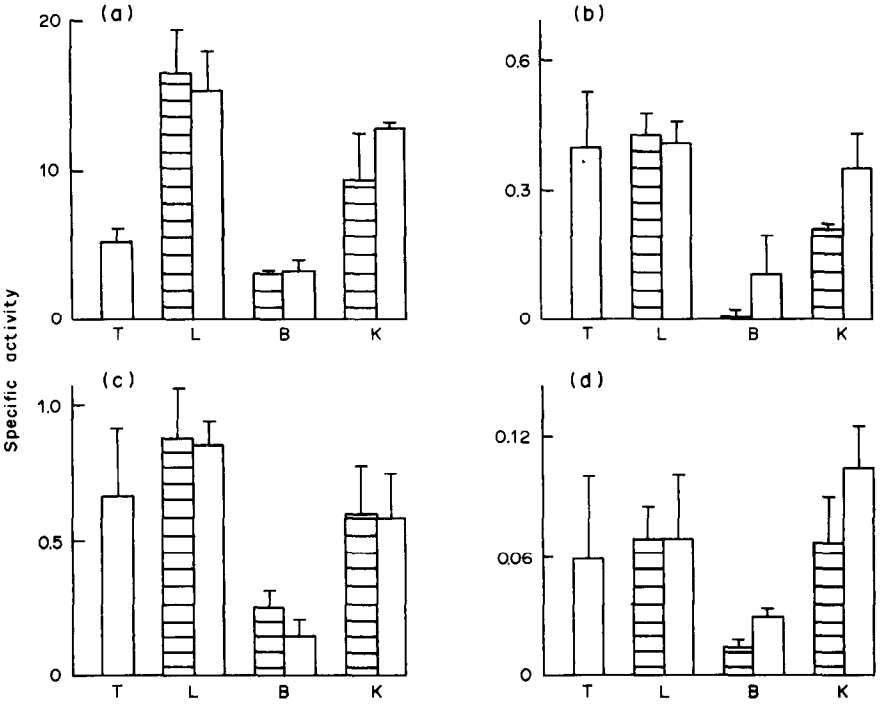

Fig. A1. Activity levels of ALA/D (a), PBGase (b), HMB-S (c) and URO-D (d) in different mouse tissues. T, Tumor; L, liver; B, brain; K, kidney. □, Normal mice; ▤, tumor bearing mice. Each value represents the mean ± SD obtained from 5 different mice of each type. Experimental details as well as enzyme units are described in the text.

In analogy with PBGase, tumoral HMB-S also showed values similar to those of liver and kidney [Fig. A1(c)]. As to the ratio between HMB-S and PBGase it was nearly 2 and approximately the same in all the studied tissues.

Tumoral URO-D activity as already discussed for PBGase and HMB-S was rather the same of that of liver and kidney [Fig. A1(d)].

To the best of our knowledge studies on these enzymes in tumors are scarce and were carried out only in human specimens. Significant diminished ALA-D activity was found in a variety of tumors from human gut, when compared with normal tissues (Rasetti *et al.*, 1967). However these same authors observed increased porphobilinogen biosynthesis in tumor tissues from lung and uterus as compared with the corresponding normal specimens (Rasetti *et al.*, 1967).

In leukocytes from patients suffering either acute or chronic myelocytic leukemia 3–5-fold decreased ALA-D activity was detected in contrast with normal and mature lymphocytes and granulocytes (Takaku and Wada, 1968).

HMB-S activity from peripheral lymphocytes was higher in patients with malignant lymphoproliferative diseases such as chronic lymphocytic leukemia and lymphoma than in normal individuals (Lahav *et al.*, 1987).

Taking into account the above results coming from our and other groups, we conclude that a partially altered enzyme pattern could exist in neoplastic tissue as compared with a high heme producing tissue such as liver. Changes observed for ALA-D activity were quite different depending on the original tissue. It is interesting to note that HMB-S activity seems to be enhanced in all neoplastic tissues examined so far. Furthermore, the results reported here are also in agreement with studies carried out with human breast carcinoma.