BIOCHEMICAL STUDIES ON ACID AND ALKALINE RIBONUCLEASE IN SERA OF WOMEN WITH DIFFERENT OVARIAN TUMORS

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Abstract

Acid and alkaline ribonuclease (RNase) activities have been assayed in (14) serum samples of women with histological confirmed ovarian carcinoma, (26) samples of women with benign ovarian tumors, and (27) serum samples of healthy women without any clinically detectable diseases. The mean values of acid and alkaline RNase specific activities were statistically increased in the cancer group ($p<0.001$) in comparison with those of other groups. The ratio of acid to alkaline RNase (Ac/Al) activities was statistically decreased in the cancer group as compared with those of other groups.

Serum alkaline RNase levels were estimated in sera of the above mentioned groups using bovine pancreatic RNase (A), as a standard. A markedly increase in this level in sera of the cancer group was observed when matched with those of other groups.

Different alkaline RNase forms were detected in sera of control, benign and malignant tumors groups using gel filtration chromatography (1.6 × 60 cm. Sephadex G75 column). The results indicate the presence of five different forms in each case, which were different in their molecular weights.

Introduction

Nowadays there is an increasing interest in serum factors that are related qualitatively or quantitatively to the presence of neoplasm in humans. Factors both specific for certain tumors e.g., catecholamines, and non–specific alkaline phosphatase, have proved useful for the detection of tumors and monitoring the course of disease. Recently correlations between the presence of tumors and blood levels of a growing list of substances, called tumor markers, have demonstrated the value of assays that, more sensitively, reflect tumor status than conventional tests. These tumor markers are classified to:

1) Tumor–specific tumor markers (Carcino Embryonic antigen, carbohydrate 19–9, heat stable alkaline phosphatase, and tissue poly peptide antigen).
2) Tumor–associated tumor markers (Ferritin, the ratio of ferritin to serum iron, immune suppressive acidic protein, and sialic acid).
3) Growth–related tumor markers (alkaline phosphatase isoenzymes, ribonuclease). [1]

Two types of RNase (Ec : 3.1.4.22) activities have been known to exist in human serum; acid and alkaline RNase [2], that differ in their: pH optimum, affinity for synthetic substrate, and in absolute requirement for divalent cation. [3, 4]

RNase activity is present in most mammalian cells in both the free form and bound to the naturally occurring inhibitor protein , which is a (50) KD protein that [5] and constitutes (0.01%) of the cytosolic protein. The equilibrium between the RNase and its inhibitor may have a role in regulation of the turnover of cytoplasmic protein synthesis, and its function is to preserve the integrity of cellular RNA when secretory RNase reaches the cytosol. [6]

It is generally accepted that the biological role of RNases is not known. Nevertheless, it could be suggested that these enzymes might be considered as factors, which protect the genetic stability of the cell [7].

It has been reported by a number of authors that the levels of RNase activities in human body fluids, such as serum and urine, may be an indicative of disease state. ([8, 9], in previous work carried out in our laboratory, sera acid and alkaline RNase activities of patients with uterine tumors show a highly significant elevation ($p < 0.001$) in comparison with those of control women and benign uterine patients. [10]

The aim of the present work is to investigate the clinical usefulness of RNase activities in ovarian cancer patients and follow the changes in its different forms upon malignancy.

Materials and methods:

Chemicals

All chemicals used throughout this work are of analar grade.

Patient's samples:

Five milliliters of blood samples were collected from (27) healthy women to be used as control, while samples that used as test groups were collected from (26) patients with benign ovarian tumors, and from (14) ovarian cancer patients. All patients were admitted for management to Al-Elweia teaching hospital, and Al-Arabee private hospital.

The diagnoses were proven by cytological histopathological examination in these hospitals. Any patient with coexisting diseases was excluded. Samples of each mentioned groups were subdivided into pre–, meno-, and post menopausal women. The blood was allowed to coagulate at room temperature before being centrifuged at (3000×g) for (10) minutes, the resultant sera were separated and stored at (-20) ^{o}C until used.

Total protein determination:

Serum total protein was determined by Lowry *et. al.* method[11] using bovine serum albumin (BSA) as a standard protein.

Determination of acid RNase activity:

Acid RNase activity was estimated according to Smith *et. al.* method [12] . Where (0.1) ml of serum was added to a mixture containing (0 .1 %) RNA solution, used as substrate, dissolved in acetate buffer (0.1 M ؛ pH 5.0), and the total volume of the mixture was completed to (3) ml with double distilled water.

The rate of the increase in absorbance at (300) nm was followed against the blank, for (2) minutes at (25) ^{o}C.

Determination of alkaline RNase activity and its level:

Alkaline RNase activity was determined using Chretion *et. al.* [13] modified method. The reaction mixture contains the following solutions, with final volume of (1) ml: Tris – HCl buffer (0.2 M; pH 8.5), RNA solution (1%), and diluted serum with phosphate buffer (0.1 M; pH 6 .7) (1:5). This mixture was incubated for (20) min. at (37) ^{o}C, cooled to (4) ^{o}C, and mixed with an equal volume of HCl (1) M in (70 %) ethanol. After (1) h, the samples were centrifuged. The supernatant was diluted (1:10) with distilled water, then the absorbance at (260) nm was measured. If the absorbance was beyond the limits (0.14–0.38), more serum dilution was made.

The Level of alkaline RNase was determined by substituting the serum in the above procedure with (0.4 ml) of standard RNase (A) solution (1 µg/ml) where each (0.1) A unit is equivalent to (0.0027) mg of crystalline pancreatic RNase [13] .

Expression of enzymes activities:

For all determined enzymes, International unit was used to express enzymes activities, which is defined as the amount of the enzyme that catalyzes the hydrolysis of one micromole of substrate per minute per liter of serum under optimum conditions. Enzyme activity was calculated by the following equation:

$$\text{Activity (U/L)} = \Delta A/\text{min.} \times \frac{V_t}{V_s} \times 1000$$

Where:

ΔA = (A) sample – (A) Blank.

Vt = Total volume.

Vs = Sample volume.

t = Incubation time.

While their specific activities were expressed as enzyme unit per mg of protein.

Statistical analysis:

The obtained results of this work were submitted to a computerized statistical treatment. Number of cases, maximum and minimum values, mean, error and standard deviation were calculated for each group. The data were compared by student's t-test.Differences were considered significant at ($p<0.001$).

Chromatographic separation of sera different alkaline RNase forms:

Gel filtration chromatography was used to separate serum alkaline RNase different forms following AKagi et. al. [14] method, Sephadex G-75 column (60 × 1.6) cm with bed volume

of (120.6) cm^3 was used for the separation step. The packing of the column was checked using blue dextran, where the void volume was determined and found to be equal to 38.25 ml. A volume of (2) ml of serum (40 mg protein / ml) was applied onto the surface of the gel, fractions of (4.25) ml were collected using fraction collector and Tris–HCl buffer (0.02M; pH 8.5) containing NaCl (0.5) M, as the elution buffer at flow rate of (22.5) ml /h. The protein content of each fraction was monitored by measuring absorbencies at (280) nm. RNase activity and protein concentration in each fraction were assayed using Chretion et. al. [13] method and Lowry's method [11] respectively.

Molecular weights of alkaline RNase different forms were determined using gel filtration chromatography where the following standard proteins were used: Albumin (67 KD) Ovalbumin (43 KD), Trypsin inhibitor (20 KD), and Insulin (6 KD). The molecular weight of each isolated alkaline RNase form was estimated from the calibration curve Fig.(1) of different standard protein Kav values vs. their log M.wt.. Kav were calculated as follows:

$$Kav = \frac{V_e - V_o}{V_t - V_o}$$

Where:

Ve = Elution volume of the protein.

Vo = Column void volume.

Vt = Total bed volume.

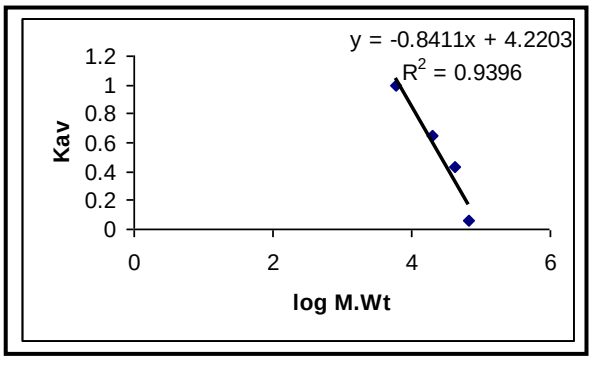

Fig. (1): Calibration curve for determination of M.wt. using gel filtration chromatography on Sephadex G-75 Column (60×1.6) cm, flow rate (22.5) ml/h, fraction vol. (4.25) ml & a void volume of.(38.25)ml as determined by the use of Blue dextran.

Results

Two types of RNase : Acid and alkaline RNase exist in human sera. The specific activities of these two RNase were estimated in sera of women with benign and malignant ovarian tumors in comparison to that of control women, where the pH optimum was used as the base to distinguish between the activities of the two types of the enzyme.

The results in Table (1) revealed a highly significant increase ($p<0.001$) in the serum acid RNase specific activity of the cancer group when matched with those of other groups. The results indicated that patients group with ovarian cancer had the highest mean value (0.167 ± 0.0016) U/mg when compared with that of benign tumors (0.1167 ± 0.003) U/mg and with that of control women (0.127 ± 0.009) U/mg.

Serum alkaline RNase specific activity was determined in the same above mentioned groups and the results show a highest mean value in the cancer group (3.28 ±0.332) U/mg with a highly significant difference when compared with that of benign tumor group (1.07 ± 0.18) U/ mg , ($p< 0.001$) and that of control group (1.035 ± 0.007) U/mg, ($p<0.001$) , as shown in Table (1).

Table (1)
Specific activity of sera acid and alkaline RNase of control, benign and malignant ovarian tumors.

				Specific activity U/mg mean (± SD)	
Group	**No.**	**Age (Year)**	**Total protein g/dL**	**Acid RNase**	**Alkaline RNase**
Control	27	19-73	7.12 (±0.87)	0.127 (±0.009)	1.035 (±0.007)
Benign ovarian tumor	26	20-70	7.33 (±0.34)	0.116 (±0.003)	1.07 (±0.18)
Ovarian cancer	14	18-71	7.21 (±0.25)	0.167 (±0.0016)	3.28 (±0.332)

Estimation of serum alkaline RNase level show a significant elevation in ovarian cancer patients (0.136 ± 0.013) µg/ml when matched with that of benign tumor (0.0765±0.037) µg /ml ($p<0.001$)and with that of control subject (0.055 ± 0.015) µg /ml, ($p<0.001$), as shown in Table (2).

Table (2)
Alkaline RNase level and acid RNase activity/ Alkaline RNase activity (*Ac /Al)ratio of sera control, benign and malignant ovarian tumor.

Group	No.	Alkaline RNase level µg/L	(*Ac /Al) ratio
Control	27	0.055 (±.015)	0.122
Benign ovarian tumor	26	0.0765 (±0.037)	1.09
Ovarian cancer	14	0.136 (±0.013)	0.0509

When the specific activities of acid and alkaline RNase were compared among the studied subgroups, the results show no differences in their specific activities between the pre–, menopausal, and post menopausal women of each group under study.

Many studies refers to the uses of acid / alkaline RNase ratio as an additional method for diagnosing, assessing the remission, and recurrence of some types of acute leukemia [15] .Our results, as seen in Table (2), revealed a decrease in this ratio in cancer group as compared with those of benign tumor and control women groups.

In order to detect any changes in alkaline RNase forms that may accompanied transformation of the cells into cancer cells, gel filtration (Sephadex G - 75) was used to separate these forms.

Figs. (2, 3, 4) shows that the enzyme present, in healthy individuals, in five different forms. Also still five forms are present in sera of the benign and malignant tumor groups.

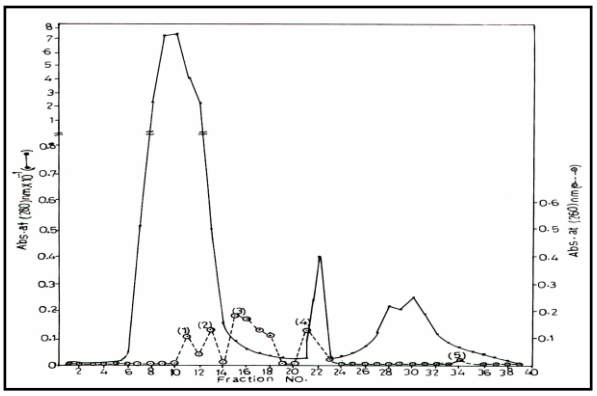

Fig.(2): Gel filtration profile of alkaline RNase forms isolated from sera of ovarian cancer patients using Sephadex G-75 column (60×1.6) cm, flow rate (22.5) ml/h, fraction vol. (4.25) ml & the void volume, as determined with blue dextran, was (38.25)ml.

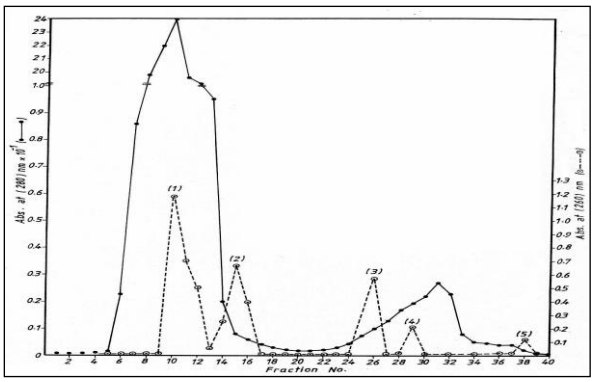

Fig.(3) : Gel filtration profile of alkaline RNase forms isolated from sera of benign ovarian tumor patients using SephadexG-75column(60×1.6) cm, flow rate (22.5) ml/h, fraction vol. (4.25) ml,& a void volume of (38.25) ml.

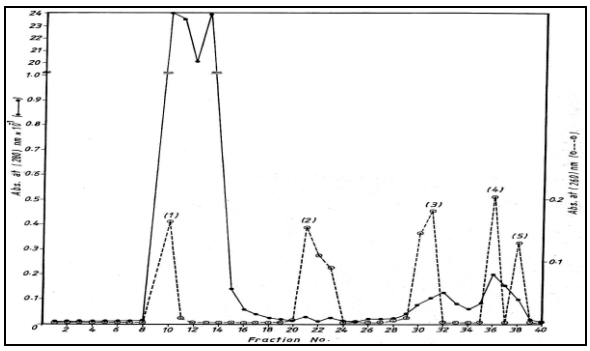

Fig.(4): Gel filtration profile of alkaline RNase forms isolated from sera of control women using Sephadex G-75 column(60×1.6) cm, flow rate (22.5) ml/h, fraction vol. (4.25) ml,& avoid volume of (38.25)ml .

but these forms have different specific activities Table (3 A, B, C) and molecular weights, as shown in Table (4).

Table (3)
Isolation of serum RNase forms from crude individual (A) and patients with benign (B) and malignant ovarian tumors (C).

Control group(A)	Protein (mg/L)	Total activity U/L	Specific activity U/mg
Crude Forms	35250	41375	1.173
1	737.92	3500	4.743
2	40.97	7500	183.06
3	70.27	8500	120.96
4	100.32	14500	144.53
5	66.82	3000	44.89

Benign ovarian tumor(B)	Protein (mg/L)	Total activity U/L	Specific activity U/mg
Crude Forms	48750	56250	1.15
1	852.35	20250	23.75
2	69.25	10500	151.62
3	76.18	15750	206.74
4	109.36	5500	50.29
5	27.89	3025	108.46

Ovarian cancer(C)	Protein (mg/L)	Total activity U/L	Specific activity U/mg
Crude Forms	42614	145000	3.40
1	749.48	27000	36.02
2	424.07	32250	76.04
3	69.28	45750	660.36
4	23.09	33000	1429.19
5	2.0994	5000	2382.08

Table (4)
Molecular weights of serum RNase forms isolated from control, benign and malignant ovarian tumors as determined by gel filtration chromatography (Sephadex G-75).

	Control	Benign Ovarian tumor	Ovarian cancer
Forms	M.wt. (D)		
1	67000	67000	67000
2	29600	30800	59100
3	21900	25900	45750
4	16700	16700	29600
5	7350	10800	7350

Discussion

Many authors has reported elevated serum levels of ribonucleases activities in patients with various types of cancer [10, 14-17], yet the expression and secretion of ribonuclease by these deferent tumors has not been documented [7].

The finding in this study of serum RNase elevation in ovarian cancer patients Table (2) is in agreement with our previous study on uterus cancer patients [10] and with many other studies on different kinds of cancers.[10,14,15,18-21].

Alterations in nucleic acid metabolism have been demonstrated in a variety of malignant conditions in addition to the hyperuricemia that often observed in some malignant conditions, which may reflect in part the increased synthesis of RNA in the tumor cells, and consequently increases in the enzyme level [22, 23].

Evidence was presented by some investigators [16], showed that serum content of RNase was a resultant of the elaboration of the enzyme by white cells. Other possibilities are:- its secretion by tumor cells or the surrounding tissues [10], and its excessive entry into serum rather than to diminish its urinary excretion [7].

The cause of elevated serum RNase levels observed here is not clear, this increase may be associated with a host defense mechanism , production by malignant cells, a secondary destructive process in other cells or tissue [7], oxidation of a single cysteine residue in the RNase inhibitor (RI), that leads to rapid oxidation of the remaining cysteine residues , and hence inactivation of RI, and consequently enzymatic reactivation [5].

Studies which showed that RNase can alter immune responsiveness may have relevance to the elevated serum RNase levels in cancer patients [6].

Alteration of the expression of carbohydrate structure is frequently observed in tumor cells, and different changes of O- , and N–linked glycoprotein observed in cancer cells [24, 25]. Since RNase is known as glycoprotein [17], differences in molecular weights of RNase forms is expected between those of ovarian cancer patients and those of normal women.

Enzyme levels may be a useful indicator of response to chemotherapy [26]. Testing this hypothesis will be the basis of the future work.

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الخلاصة

قيست فعالية انزيم RNase بنوعية الحامضي والقاعدي في مصول (14) عينة لنساء مصابات بسرطان المبيض و (26) عينة لنساء مصابات باورام المبيض الحميدة وتمت مقارنتها مع (27) عينة لنساء اصحاء . اظهرت النتائج وجود زيادة معنوية في الفعالية النوعيه لانزيم RNase الحامضي والقاعدي في مجموعة سرطان المبيض مقارنة مع تلك للمجاميع الاخرى . كما اظهرت الدراسة بان نسبة فعالية انزيم RNase الحامضي الى فعالية انزيم RNase القاعدي (Ac/Al) قد انخفضت بشكل معنوي في مجموعة سرطان المبيض عن للمجاميع الاخرى .

تم تعيين مستوى انزيم RNase القاعدي في مصول المجاميع تحت الدراسة باستخدام انزيم Bovine pancreatic RNase القياسي حيث وجدت زيادة ملحوظة في مستوى الانزيم في مصول مجموعة سرطان المبيض بالمقارنة مع تلك للمجاميع الاخرى.

شخص وجود خمسة اشكال مختلفة من انزيم RNase القاعدي في مصول النساء السليمات و المصابات باورام المبيض الحميدة و الخبيثة باستخدام كروموتوغرافيا الترشيح الهلامي [عمود سفادكس G-75 بالابعاد [1.6 × 60].سم. وعند تعيين اوزانها الجزيئية اتضح انها تختلف في العينات المرضية عما هي في عينات السيطرة.