SHORT COMMUNICATION

Serum Alkaline Deoxyribonuclease Activity, a Sensitive Marker for the Therapeutic Monitoring of Cancer Patients: Methodological Aspects

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Summary: Alkaline deoxyribonuclease (DNase) is present in human circulating serum but its physiological role and significance of its variations are still largely unknown. The present report demonstrates that between 37 °C and 50 °C, as measured in the presence of 0.25 mmol/l Ca\(^{2+}\) and 5 mmol/l Mg\(^{2+}\), serum alkaline DNase activity increases, in most sera, reaching a level far higher than expected from thermal activation. This observation is thought to be due to the thermal inactivation of a serum inhibitor of the enzyme, which limits its usefulness as a therapeutic marker. By measuring serum alkaline DNase activity at 50 °C, the authors have developed a clinical test which has been successfully applied to the therapeutic monitoring of patients with various types of cancers.

Introduction

Serum alkaline deoxyribonuclease activity has been measured in several diseases, mainly in cancer patients (1—3). Due to differences in the methods used, the low level of enzyme activity as assayed by these methods, and large interindividual variations in control levels (4), the results of such measurements were difficult to interpret. The enzyme is therefore not considered useful for the therapeutic monitoring of disease(s).

The aim of the present report is to demonstrate that between 37 °C and 50 °C, serum alkaline deoxyribonuclease activity increases, reaching in most sera a level which is much higher than expected from the observed thermal activation of purified alkaline deoxyribonuclease. Such an original observation led us to develop a new and sensitive assay for serum alkaline deoxyribonuclease activity based on the quantitative measurement of soluble oligonucleotides liberated from DNA hydrolysed at 50 °C in the presence of a serum sample. This assay has been used, with success, in the therapeutic monitoring of cancer patients (5—10) and has been confirmed on experimental tumours in rats (11).

Materials and methods

A highly polymerized DNA from calf thymus (Sigma, St Louis MO, USA) was used as substrate for measuring alkaline deoxyribonuclease (DNase) activity. Bovine pancreatic DNase (DNase I) was purchased from Sigma, St Louis MO, USA. All other chemicals used were of the purest analytical grade available from E. Merck, Darmstadt, Germany.

Serum samples were obtained by centrifuging coagulated venous blood, collected in dry tubes to avoid chemical anticoagulants such as EDTA, NaF or citrate which may interfere with the assay. These sera were kept frozen at −20 °C until use. Under these conditions serum alkaline deoxyribonuclease activity is stable for more than 6 months.

Assay conditions

Alkaline deoxyribonuclease activity was assayed by spectrophotometric measurement of the acid-soluble oligonucleotides liberated from DNA, using an adaptation of the method of Loiselle & Carrier (2). Activity is expressed as kU/l, using an average molar lineic absorbance of 880 m\(^{2}\)/mol. Total volume of the incubation solution was 1 ml, consisting of 0.1 ml of test serum and 0.9 ml of 0.1 mol/l Tris buffer pH 8 containing 500 g/l of DNA, 5 mmol/l MgCl\(_2\) and 0.25 mmol/l of CaCl\(_2\). Each blank had the same components as the test, but also contained 10 mmol/l EDTA to inhibit alkaline DNase activity by the chelation of Ca\(^{2+}\) and Mg\(^{2+}\) ions. Both tubes (test and corresponding blank) were incubated for 1 h in a water bath at 50 °C. At the end of the incubation, the tubes were transferred to an ice bath. The enzymatic hydrolysis was stopped by adding 0.5 ml of a saturated solution of MgSO\(_4\) and 1.5 ml of ice-cold 2.25 mol/l perchloric acid to precipitate non-hydrolysed poly-nucleotides. After at least 30 min in the ice bath to allow precipitates to form, the tubes were centrifuged at 2000 g for 20 min and the absorbance at 260 nm was measured in the supernatants. To determine the optimal incubation conditions as described above, which is the principal aim of the present investigation, the following variations were examined: DNA concentrations from 50 to 1000 mg/l; MgCl\(_2\) from 0.5 to 50 mmol/l; CaCl\(_2\) from 0.1 to 5 mmol/l; temperature of incubation from 37 °C to 60 °C.

1) Enzyme: Alkaline deoxyribonuclease: Deoxyribonuclease I (EC 3.1.21.1)
Results

Effect of increasing concentrations of Mg$^{2+}$, Ca$^{2+}$ and DNA on DNase I activity

A solution of commercial DNase I (30 μg/l) was incubated under the experimental conditions described in Materials and Methods for 1 h at 37 °C in the presence of 500 mg/l DNA and different concentrations of Ca$^{2+}$ and Mg$^{2+}$ ions. Maximal activity of the enzyme was observed at concentrations between 2 to 10 mmol/l for Mg$^{2+}$ and at 0.25 mmol/l of Ca$^{2+}$. In all further assays, the concentrations of Mg$^{2+}$ and Ca$^{2+}$ were 5 and 0.25 mmol/l respectively.

The enzyme was then measured in the presence of increasing concentrations of DNA, in an experiment which showed that the enzyme displayed Michaelis and Menten type kinetics and that saturation of the enzyme was reached at a concentration of 400 mg/l of DNA (fig. 1). In all further assays, the concentration of DNA was 500 mg/l.

Effect of temperature of incubation on DNase I activity

Under the experimental conditions defined above, DNase I was incubated at temperatures varying from 37 °C to 60 °C. Under all these conditions, the enzyme behaved similarly with regard to ions or substrate concentrations. Maximal activity was observed at 53 °C, and above this temperature the enzyme was inactivated (fig. 2). The ratio of activity at 37 °C to that at 50 °C is 1.95 ± 0.03.

Assay of alkaline DNase activity in human sera

All the experimental conditions defined for the DNase I assay were found to be optimal for serum alkaline deoxyribonuclease activity. Under these conditions, the level of human serum alkaline deoxyribonuclease activity measured at 37 °C after 1 h of incubation was usually very low (0.2 to 6.3 kU/l). But after incubating human sera at temperatures varying from 37 °C to 70 °C a maximal activity was observed between 52 and 55 °C for each serum tested, and total inactivation of the enzyme occurred at 70 °C. By using serum from healthy donors and cancer patients it was found that the activity varied between subjects and that the calculated ratio between the activity measured at 50 °C and the activity at 37 °C differed between sera and was without any correlation with the level of the activity at 37 °C (tab. 1). This ratio varied from 5 to 80, but it was in all cases higher than that determined for purified DNase I (1.95 ± 0.03).

By plotting enzyme activity vs temperature according to Arrhenius' law it was shown that the natural logarithm of DNase I activity (fig. 3a) vs 1/T follows a straight line corresponding to an apparent enthalpy of activation of 42 kJ/mol (10 kcal/mol) up to the inactivating temperature. For serum alkaline deoxyribonuclease activity (fig. 3b) such a relationship breaks down at approximately 45 °C, and these data were interpreted as showing a change in the apparent enthalpy of activation from about 252 kJ/mol (60 kcal/mol) up to 84 kJ/mol (20 kcal/mol).

These data support the hypothesis that the increase of serum alkaline deoxyribonuclease activity between 37 °C and 50 °C is not only due to the thermal activation of the enzyme but probably to the inactivation of a thermolabile factor which inhibits most of the circulating enzyme (12).

A temperature of 50 °C was chosen for all further determinations of serum alkaline deoxyribonuclease activity. Under the chosen conditions (5 mmol/l Mg$^{2+}$, 0.25 mmol/l Ca$^{2+}$, 500 μg DNA, 1 h and 50 °C) the intra-assay coefficient of variation (CV) was 3.6% for a mean activity of 16.3 kU/l and 4% for a mean activity of 41.6 kU/l. The inter-assay CV determined by analysing two control serum specimens in 10 different assays was 8.5% for a mean activity of 16.7 kU/l and 3.1% for a mean activity of 43.1 kU/l.

Alkaline DNase activity of human serum and plasma: Comparison and stability

Some authors (13) have shown that the level of alkaline DNase is higher in serum than in plasma, and have therefore proposed that the enzyme is released from platelets during clotting. Alkaline DNase activities were therefore measured in the serum and plasma from five normal individuals using the experimental conditions described in Materials and Methods. Furthermore, the stability of serum alkaline deoxyribonuclease activity was examined by allowing the blood to clot at room temperature for various lengths of time before serum was separated. Table 2 shows that there was practically no difference between serum alkaline deoxyribonuclease activity and the DNase activity of heparinized plasma. Serum alkaline deoxyribonuclease activity remained stable in clotted blood for at least five days (data not shown) as well as in serum samples kept frozen at −20 °C for at least 6 months.

Serum alkaline deoxyribonuclease activity level in healthy people and untreated cancer patients

Serum alkaline deoxyribonuclease activity was measured in 200 randomized healthy individuals (fig. 4a). A wide distribution of serum alkaline deoxyribonuclease activity levels was observed between individuals (mean value: 23 ± 10 kU/l, range: 4—54) but without day-to-day variations for one person.

Important variations of serum alkaline deoxyribonuclease activity levels were observed during and after cancer treatment,
when the assays were performed at 50 °C; whereas at 37 °C variations were so small that it was extremely difficult to analyse them. Figure 5 shows the variations in the level of serum alkaline DNase activity in a patient with acute non-lymphoblastic leukaemia before, during and after treatment.

Discussion and Conclusions

The method used in the present work and in our previous studies for the assay of human serum alkaline deoxyribonuclease activity is based on the original observation that when sera are incubated at 50 °C there is significant increase in serum alkaline DNase activity compared with the assay at 37 °C. This increase varies considerably from person to person.

Throughout the therapeutic monitoring of cancer patients, serum alkaline deoxyribonuclease activity at 37 °C showed no significant variations. However, serum alkaline deoxyribonuclease activity measured at 50 °C shows typical variations which correlate with the evolution of the disease.

Further studies indicate that this increase is not due simply to thermal activation, and it appears that a heat-labile factor is present, which interacts either with the enzyme or with the substrate and influences the assay at 37 °C.

We therefore propose that expression of the almost total activity of the circulating enzyme is prevented by a thermostable inhibitor, and provided this is inactivated or destroyed, the variations in the resulting activity reflect the evolution of the tumour.

The presence of natural specific inhibitors of alkaline DNase has been well reported in normal and cancerous tissues of animal origin (14—16). Lazarides & Lindberg (17) identified this inhibitor as actin, one of the main components of the cytoskeleton. In human serum, only a few reports describe a heat labile inhibitor of DNase I. These reports suggest that the inhibitor is released from white blood cells (18,19) and platelets (20) and that it is inactivated by heating at 56 °C for 10 min.

However, the inherent activity of DNase I in serum was not determined in any of these reports, so it was unclear whether DNase inhibitors affected the endogenous serum enzyme.

Various modulators and more or less complicated mechanisms can be proposed to account for the typical serum alkaline deoxyribonuclease activity variations during therapeutic monitoring of cancer patients. Two possible mechanisms are:

1. uptake of circulating serum alkaline DNase by cancer cells, which are very rich in DNase inhibitors, or

2. release of a thermostable alkaline DNase inhibitor into the circulation by the cancer cells, whose membranes are permeable, especially when destroyed by efficient therapy.

Since absolute serum alkaline deoxyribonuclease activity values in untreated cancer patients are not dramatically different from those in normal individuals, they cannot be considered as a valuable diagnostic test for cancer. However, serum alkaline deoxyribonuclease activity variations periodically measured during and after the therapy and compared with the pretreatment value for each patient appear to be a useful biological marker for monitoring the response to treatment and for predicting the long-term evolution of cancer pathology. Serum alkaline deoxyribonuclease activity also appears to be useful for detecting recurrence of disease before the appearance of clinical signs of relapse, as observed by us in acute non-lymphoblastic leukaemia patients (8).

Clinical results from more than 600 patients with miscellaneous cancers were previously published by our group of investigators. They showed (fig. 6) that when the analysis is performed at 50 °C:

— Serum alkaline deoxyribonuclease activity decreases significantly a few days after therapy (phase I), provided the treatment is effective. In the absence of a clinically verified response to treatment (5—7, 9—10) there were no variations in serum alkaline deoxyribonuclease activity;

— weeks after treatment (phase II), complete clinical remission was accompanied by a progressive increase in serum alkaline deoxyribonuclease activity until it exceeded its pretreatment value. An incomplete recovery of the serum alkaline deoxyribonuclease activity value was correlated with partial tumour regression. No increase of serum alkaline deoxyribonuclease activity was observed in cases with an initial minor response, followed by a fatal evolution (5—7, 9—10);

— months after treatment (phase III in acute non-lymphoblastic anaemia), the stabilization of the serum alkaline deoxyribonuclease activity level reached at complete remission indicated the continuation of remission. A distinct and continuous decrease in serum alkaline deoxyribonuclease activity without any treatment revealed an early recurrence and/or occult disease not otherwise detectable by routine investigation at that stage. Such detection of recurrence by serum alkaline deoxyribonuclease activity preceded the clinical diagnosis by 3 weeks to 5 months (8).

Fig. 5. Variations of alkaline DNase activity in the serum of an acute non-lymphoblastic leukaemia patient during therapeutic monitoring. Measurements at (a) 37 °C, (b) 50 °C.

Fig. 6. Synthetic profile of the evolution of serum alkaline DNase activity versus clinical response in patients with malignant tumour. T0 is serum alkaline deoxyribonuclease activity level of the patient at the time of initial diagnosis, before therapy.

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References
