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Neoplastic Characteristics of the DNA Found in the Plasma of Cancer Patients[1]

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Abstract. About one third of patients with various malignant diseases were found to have extractable amounts of DNA in their plasma whereas no DNA could be detected in normal controls. Using the test established by one of us (M.B.), which is based on decreased strand stability of cancer cell DNA, we have found that several plasma DNA originate from cancer cells.

Introduction

Increased plasma levels of DNA have been observed in cancer patients. These results have been obtained either by indirect methods such as radioimmunological or actinomycin-D-binding tests [1–3] or by direct purification of DNA from the plasma [4]. In this last study the DNA was characterized as heterogenous being composed of fractions ranging from 21 kilobases (kb) to less than 0.5 kb. All these fractions hybridized with a human DNA probe, indicating the human origin of the bulk of the circulating DNA. However, it could not be determined if the circulating DNA was released from activated host lymphocytes [5] or from the tumor cells themselves.

One way to solve this problem would be to take advantage of differences between certain properties of the DNA of malignant and normal cells, and to find out if the DNA isolated from the plasma of cancer patients presents some of the characteristics recently detected in the DNA of malignant cells [6]. Experiments performed by one of us (M.B.) have indeed demonstrated that DNA extracted from a variety of neoplastic cells differs from its normal counterpart by some specific properties which will be briefly mentioned. Upon exposure to certain chemical carcinogens or to anticancer drugs with known carcinogenic activity (referred to as 'carcinogens' in the text), DNA strand separation measured by the increase in ultraviolet (UV) absorbance (hyperchromicity) appears at lower temperature, suggesting a decreased strand stability in the malignant state. The same carcinogens at given concentrations stimulate in vitro DNA synthesis in a nucleoside triphosphate DNA-polymerase-containing system much more strongly in the presence of a DNA template obtained from neoplastic cells than from normal cells. This coexistent decrease in strand stability and enhanced DNA synthesis in the presence of carcinogens have been found for all malignant tissues tested so far. In the present work we used the same techniques for the study of the DNA found in the plasma of cancer patients. These findings have been confirmed by other authors [7, 8].

Materials and Methods

Patients and Material

Forty-milliliter blood samples were collected on heparin from a total of 56 patients with various advanced malignancies who were not receiving any kind of anticancer drugs during this period. The

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plasma was imediately separated from the blood cells, and kept at −30 C until used.

Plasma of healthy donors does not yield in the same conditions enough DNA to be extractable [4] but previous experiments from our laboratory have shown that detectable amounts of DNA appear in the plasma of whole-blood samples after a storage of more than 15 days at 4 C. This is why we extracted DNA from the plasma of whole blood of healthy donors stored for 30 days and used this material as control. This finding also guarantees that when the plasma is immediately separated from the blood cells any plasma DNA of cancer patients cannot be due to spontaneous cytolysis or to manipulation of the cells.

Plasma DNA Purification

The plasma was diluted to 60% with a solution of 0.9% NaCl and 1% SDS and shaken for 20 min in the presence of an equal volume of phenol at 60 C saturated with water. After centrifugation for 20 min at 27.000 *g* the aqueous phase was collected, mixed with 2 vol of ether and centrifuged in order to remove residual phenol. The aqueous phase was collected, shaken with an equal volume of a mixture of chloroform and isoamylic alcohol (10:1 vol vol) and centrifuged as before. After dialysis and concentration the aqueous phase was passed over a Sepharose-bound concanavalin A column to remove the high amount of polysaccharides present in the solution [9]. The eluted material was then centrifuged at 90.000 *g* on a Cs_2SO_4 gradient for 75 h at 20 C. Fractions were collected and their DNA content determined by spectrophotometry at 260 nm. The sharp peak of eluted DNA was kept and dialyzed.

In vitro DNA Synthesis Test

The incubation mixture for in vitro DNA synthesis contained per 0.15 ml: 25 µmol of Tris-HCl buffer (pH 7.65); 2 µmol of $MgCl_2$; 5 nmol of each of the four deoxyribonucleoside 5'-triphosphates (+ ^{3}H-TTP: 50.000 cpm); 0.5 µg of template DNA, and 60 µg of DNA-dependent DNA polymerase I (partly purified from *Escherichia coli*). The following carcinogenic compounds were used to test the DNA strand stability: 2-*dl*-ethionine: mitomycin C, and misulban (busulfan: 1,4-dimethanesulfonoxybutane). These agents were dissolved in water. Those with a low water solubility were first dissolved in a minimal volume of 96% alcohol and 1% deoxycholate which was then diluted with water. The pH was adjusted to 7.65. The incubation mixture was incubated for 10 min at 36 C. The reaction was stopped by the addition of trichloroacetic acid (5% final concentration) and cooled in an ice bath. Acid-precipitable material was filtered on GF C glass filter, washed four times (TCA 5%), dried with ethanol 95%, and radioactivity was measured. Increased DNA synthesis as soon as the carcinogens are added characterizes a cancerous DNA.

UV Absorbance (Hyperchromicity) of DNA

The UV absorbance at 260 nm of the different samples of DNA (10 µg in 1 ml of Tris-HCl buffer 10^{-2} *M*, pH 7.35) was measured at 24 C before and after addition of a given compound. The blank cuvette contained the equivalent amount of the same compound. DNA and the carcinogenic compound were gently shaken for 1 min. Results are expressed as UV absorbance increase in percent.

Antineoplastic Treatment of the Patients

At the time the blood samples were taken, the patients were not subjected to any antineoplastic treatment. The exact previous medical application some had is indicated for the patients whose plasma DNA was used in the in vitro synthesis test and in the hyperchromicity test.

Chemicals

Deoxyribonucleoside-5'-triphosphates (d-XTP): Miles Laboratories, USA; ^{3}H-labeled TTP (specific activity 17.5 Ci mmol): Amersham, UK; pancreatic Dnase, RNase, RNase T1: Worthington, USA; *dl*-ethionine: Biochemicals, USA; mitomycin C: Sigma, USA; misulban: Lab. Techniforma, Monaco; phenol: Merck, FRG; concanavalin A-Sepharose: Pharmacia, Sweden.

Results

Amount of DNA Found in Plasma of Cancer Patients

Different series of patients were investigated and in some of them extractable amounts of DNA were found: chronic lymphocytic leukemias 3/5, acute lymphocytic leukemias 2/3, acute myeloblastic leukemias 2/4, lung cancers 0/7, lung cancers with metastases 3/10, various abdominal tumors 1/2, prostatic cancers with metastases 2/6, pancreatic cancer 1/1, pancreatic cancers with metastases 0/2, breast cancers with metastases 0/2, kidney cancers with metastases 1/2, cholangiocarcinoma 0/1, hepatoma 0/1, ovarian neoplasia 1/1, melanoma with metastases 0/1, metastases from unknown primary tumors 1/5.

As shown in table 1, amounts ranging between 1.2 and 437 µg were isolated from 20 ml of plasma. No DNA could be extracted in 20 ml of plasma from 50 normal control subjects.

Table 1. Plasma levels of DNA in various malignant conditions

Diagnosis	DNA amount per sample, µg/20 ml		
Chronic lymphocytic leukemias	9.3	1.5	6.5
Acute lymphocytic leukemias	8.4	3.4	
Acute myeloblastic leukemias	6.5	2.6	
Lung cancers with hepatic metastases	85.5	1.2	56.6
Abdominal tumor with hepatic metastases	24		
Prostatic cancer with bone metastases	6.8	98	
Pancreatic cancer	1.3		
Kidney cancer with different metastases	106		
Ovarian neoplasia	2.2		
Hepatic metastases from an unknown primary tumor	437		

Characteristics of the DNA Purified from the Plasma of Cancer Patients

The purified DNA is double-stranded as shown by its elution characteristics on hydroxyapatite columns and its density after Cs_2SO_4 ultracentrifugation. It is resistant to RNAse, KOH and pronase, but sensitive to DNAse I. There is a correlation between its amount estimated by UV absorption at 260 nm and its amount revealed by Dische color test.

Agarose gel electrophoresis shows that the plasma DNA of cancer patients is heterogenous and composed of fractions of different sizes ranging from 21 kb to less than 0.5 kb.

Origin of DNA Found in Plasma of Cancer Patients

The tests based on the in vitro DNA synthesis in presence of carcinogens could be performed only when a sufficient amount of DNA (8–10 µg) was collected from the plasma (table 2). As shown in figure 1, 5 out of 7 plasmatic DNA samples from cancer patients presented an increased in vitro synthesis ranging from 65 to 300%. In contrast, no increase was observed with control DNA of healthy donors extracted from 9 plasma obtained from whole-blood samples stored for 30 days at 4 °C.

The in vitro DNA synthesis test was completed by the hyperchromicity test which for quantitative reasons could be performed only in 6 out of 7 samples.

Table 2. Tumor diagnosis and previous treatment of patients whose plasma DNA was subjected to the in vitro synthesis test and to the hyperchromicity test

Diagnosis	Previous antineoplastic treatment
Chronic lymphocytic leukemia	no
Lung cancer with hepatic metastases	last chemotherapy[1]
Lung cancer with hepatic metastases	last chemotherapy[2]
Abdominal tumor with hepatic metastases	no
Prostatic cancer with bone metastases	no
Kidney cancer with different metastases	no
Hepatic metastases from unknown primary tumor	no

No radiotherapy was given in any of the cases.

[1] Last chemotherapy (32 days before blood collection): cytoxan, methotrexate, oncovin, procarbazine.

[2] Last chemotherapy (34 days before blood collection): ifosfamide.

All 5 cancerous DNAs which had shown an increased in vitro synthesis presented a hyperchromic effect at room temperature when carcinogens were added. A typical example is shown in figure 2a. In contrast, the DNA isolated from the plasma of the cancerous patient which had not reacted with carcinogens in the in vitro synthesis test also showed no increase in UV absorbance at room temperature in presence of the carcinogens (fig. 2b). The 9 control DNAs presented a hyperchromic effect only in presence of KOH (typical result in fig. 2c).

In figure 3, while the cellular DNA of a leukemic patient presents cancerous characteristics, its plasma DNA is normal.

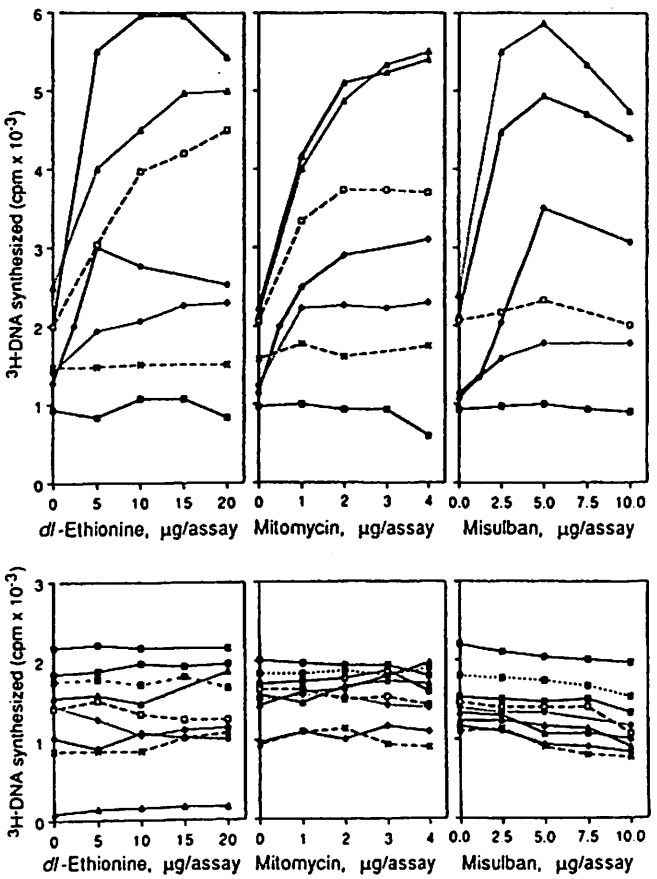

Fig. 1. In vitro DNA synthesis in presence of different carcinogens. a Plasma DNA of patients suffering from: chronic lymphocytic leukemia (X); lung cancer with hepatic metastases (◆); lung cancer with hepatic metastases (◇); abdominal tumor with hepatic metastases (■); prostatic cancer with bone metastases (△); kidney cancer with different metastases (▲), and metastases from unknown primary tumor (□). b Plasma DNA of 9 healthy donors.

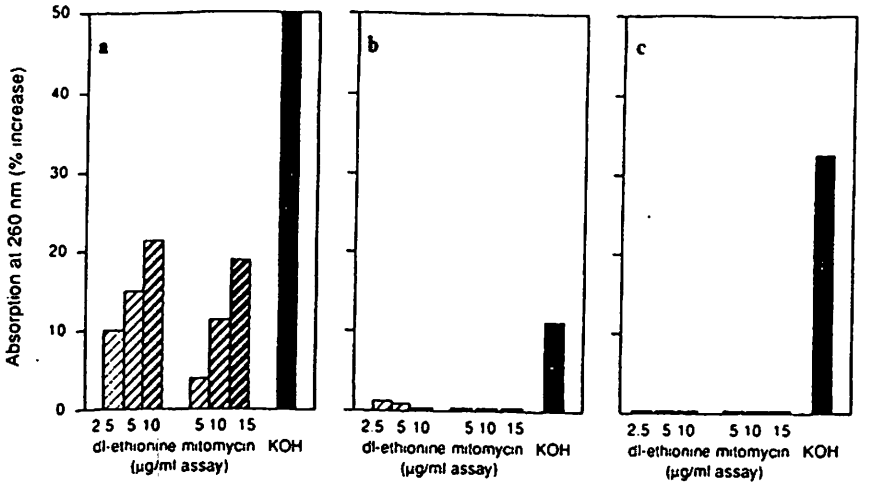

Fig. 2. Effect of different carcinogens on DNA strand separation. a Plasma DNA of a patient with hepatic metastases from an unknown primary tumor. b Plasma DNA of a patient with an abdominal tumor and hepatic metastases. c Plasma DNA of a healthy donor.

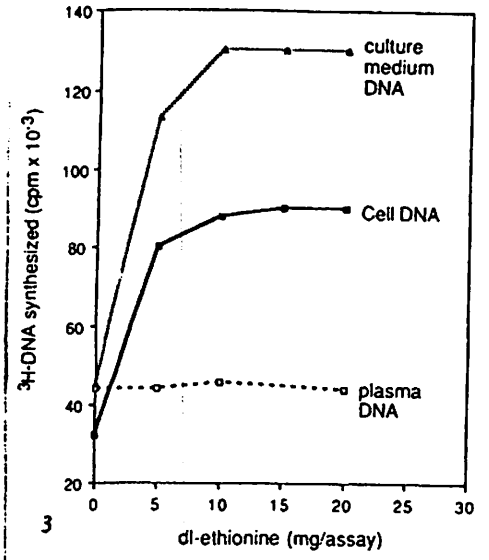

Fig. 3. In vitro DNA synthesis in presence of *dl*-ethionine.

Discussion

In 17 out of 53 (32%) patients suffering from various malignant diseases, double-stranded DNA was isolated and purified from the plasma. This proportion is very similar to the one found in a previous study [4]. Only 7 cases had a sufficient amount of plasma DNA available for in vitro synthesis tests in the presence of carcinogens. In 5 cases the tests showed the increased DNA synthesis pattern typical of neoplastic DNA, whereas in 2 cases no in vitro synthesis increase was detected, indicating the presence of normal DNA in the plasma of these cancer patients. Hyperchromicity tests which were carried out whenever a sufficient amount of DNA was available correlated always with the in vitro DNA synthesis data. As shown in figure 2 and table 1 and 2, the presence of plasmatic DNA cannot be attributed to chemotherapy or radiotherapy. Indeed, most of the cases presented had not received any previous antineoplastic treatment. Leon et al. [1] have already observed that patients responding to radiotherapy usually showed decreasing plasma DNA levels whereas increasing levels should be expected if tumor necrosis were responsible for the phenomenon.

The presence of plasma DNA of the neoplastic type in cancerous patients can be easily explained as released by the tumor cells. On the other hand, plasma DNA showing no cancerous characteristics in such patients could originate from nonmalignant host cells belonging to the immune system which during their reaction to the tumor release DNA in the bloodstream. Isolation of host DNA from the plasma has indeed been reported in patients suffering from systemic lupus erythematosus or viral hepatitis [10,11] and its presence attributed to a stage of chronic inflammation and immune stimulation. We know that normal human lymphocytes release in vitro a nucleoprotein complex requiring an active process restricted to living cells [12, 13] and that the excretion of this DNA-containing complex is enhanced by mitogenic or antigenic in vitro stimulation [5]. Such a mechanism which has been postulated in nonneoplastic diseases characterized by a state of chronic immune stimulation [10, 11] could also play a role in certain malignant situations although the conditions leading to such an in vivo DNA release by immune cells are still unclear. It is furthermore conceivable that the two mechanisms

leading to increased DNA plasma levels are not mutually exclusive and coexist in certain patients. The malignant or normal characteristics of the plasma DNA as revealed by the tests used in our study will depend on the relative amount of these two varieties of DNA each one excreted by different cells.

In order to further confirm this hypothesis, we cultured the leukemic cells of a patient whose plasma DNA showed the normal pattern and found DNA with malignant characteristics released in the culture medium.

Whatever the reasons of the presence in the blood of cancerous DNA might be, the possibility that, in some cases, it could be transfectant and thus contribute to the diffusion of the tumor cannot be discarded.

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