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Correlation between in vitro DNA Synthesis, DNA Strand Separation and in vivo Multiplication of Cancer Cells

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Abstract. The chemicals 9,10-dimethylbenzanthracene (DMBA), ethionine, daunorubicin, actinomycin D, 1-(2-chloroethyl-1)-nitrosourea (CCNU), steroids, croton oil and dimethylsulfoxide (DMSO) were used in order to correlate their effect on the in vitro synthesis of normal and cancer DNA, on DNA strand separation and on accelerated in vivo multiplication of cancer cells. All of the compounds tested strongly stimulate the synthesis of cancer DNA in vitro catalyzed by DNA-dependent DNA polymerase I and measured as an acid-precipitable labeled product. Under the same conditions, the synthesis of DNA originating from healthy tissues is only slightly enhanced, except in the case of croton oil and DMSO. These substances are almost equally active on cancer and normal DNA. Although both cancer and normal DNA contain a large amount of double-stranded regions, the extent of DNA strand separation measured by the increase in UV absorbance (hyperchromicity) in the presence of each compound tested is much higher for all cancer DNA than for corresponding normal DNA. In contrast, DMSO and croton oil do not appear to distinguish cancer DNA from normal DNA. Additive and differential effects of various compounds on cancer DNA strand separation can be observed. Small doses of DMBA and CCNU stimulate the multiplication of Ehrlich ascites tumor cells in vivo in mice. There is thus a possible correlation between DNA strand separation, DNA synthesis, multiplication and differentiation of cancer cells in the presence of the above compunds, which is different from the response of normal cells to these compounds.

Introduction

Normal cell growth and division process (at given intervals) involves physiologically regulated unwinding and separation of DNA double strands in order to allow access to DNA and RNA polymerases. Accelerated multiplication as well as differentiation of normal and cancer cells require highly increased DNA, RNA and protein synthesis whose initial step is DNA strand separation. Using the Oncotest [2] for the screening of

carcinogenic compounds, we have shown that carcinogens strongly stimulate in vitro cancer DNA synthesis, but only slightly stimulate normal DNA synthesis. Low concentrations of carcinogens can also stimulate the multiplication of cancer cells in animals [1, 3] and plants [5, 20]. On the other hand, it has been reported that actinomycin D [21] and tumor-promoting agents such as phorbol derivatives induce terminal differentiation in promyelocytic leukemia cells [18, 21] when used at very low concentrations. Dimethylsulfoxide (DMSO) which is teratogenic in animals [8, 12], also induces erythroid cell differentiation in murine-virus-infected erythroid leukemia cells [14, 28] as well as terminal differentiation of human promyelocytic leukemia cells [10]. Similar results were obtained with steroids [28]. Although morphological, biological and immunological changes induced by phorbol derivatives or DMSO have been characterized, no precise mechanism of action has been proposed.

In order to account for enhanced in vitro cancer DNA synthesis [2] and accelerated multiplication of vegetable [5, 20] and mammalian [1, 3, 21] cancer cells in the presence of carcinogens, anticancer drugs or various other compounds such as DMSO, croton oil and steroids, we attempted to establish possible correlations between in vitro cancer DNA synthesis, in vivo cancer cell multiplication and in vitro DNA strand separation catalyzed by the above compounds.

Materials and Methods

The following reagents were used: pancreatic RNase A and RNase T_1 (Worthington Inc., N.J., USA); 9,10-dimethyl-1,2-benzanthracene (DMBA; Nutritional Biochemical Co., Cleveland, Ohio, USA); dl-ethionine (Hoechst, Paris, France); Lomustine (1-[2-chloroethyl]-3-cyclohexyl-1)nitrosourea; (CNU; Bellon, Paris, France); 5-FU (Hoffmann-La Roche, Basel, Switzerland); daunorubicin (gift from Dr. *R. Maral*, Rhône-Poulenc, Ivry/S, France); estradiol and testosterone (gifts from Dr. *Ray*, Institut Pasteur, Paris, France); lauryl sulfate (Serlabo, Paris, France); DMSO (Merck, FRG); croton oil (Corporation pharmaceutique française, Melun, France); actinomycin D (Merck, Sharp & Dohme, Rahway, N.J., USA).

Animals: Swiss mice (Ifa Credo, Orleans, France).

Origin of DNA: after excision, healthy and cancerous human tissues (breast, lung, neurocarcinoma), cancer cells (Ehrlich ascites tumor cells) or normal tissue from monkey brain, mouse spleen or duck spleen were frozen at −20 °C and then gently broken down in 2 SSC solution. DNA was extracted first using phenol and then chloroform in the presence of lauryl sulfate, as previously described [2, 26]. RNA, which contaminates DNA preparations, was practically eliminated by incubation with RNase A and T_1 RNase (20 µg and 10 units/ml, respectively) for 30 min at 36 °C in 1 SSC solution. RNase was then removed by several chloroform treatments, each of which was followed by centrifugation in an SSl Sorval centrifuge (5,000 *g* for 10 min). DNA was precipitated with 2 vol of 96% alcohol, dissolved in 2 SSC solution, and dialyzed against this same solution for 24 h at 4 °C. Purified DNA (absorbance at 260/280 = 2.1) was stored at −20 °C for several months without losing its polymerized form. Before use, DNA was dialysed against distilled water for 2 h at 4 °C in order to eliminate salts which could interfere in the experiments to be carried out.

Characterization of DNA

The RNA content, determined by the orcinol reaction [4], is lower than 10%. Protein content makes up less than 1.0% [22]. The hyperchromic effect on incubation with NaOH is 30–42% for the DNA used. In the ultracentrifuge or in an alkaline sucrose gradient the material forms an essentially homogeneous peak ranging between 26 and 36 S and a small one of 2.5–4.2 S.

Isolation of DNA-Dependent DNA Polymerase I (EC 2.7.7.7)

Since DNA-dependent DNA polymerase I from *Escherichia coli* synthesizes DNA by the same mechanism as the corresponding enzyme from mammalian

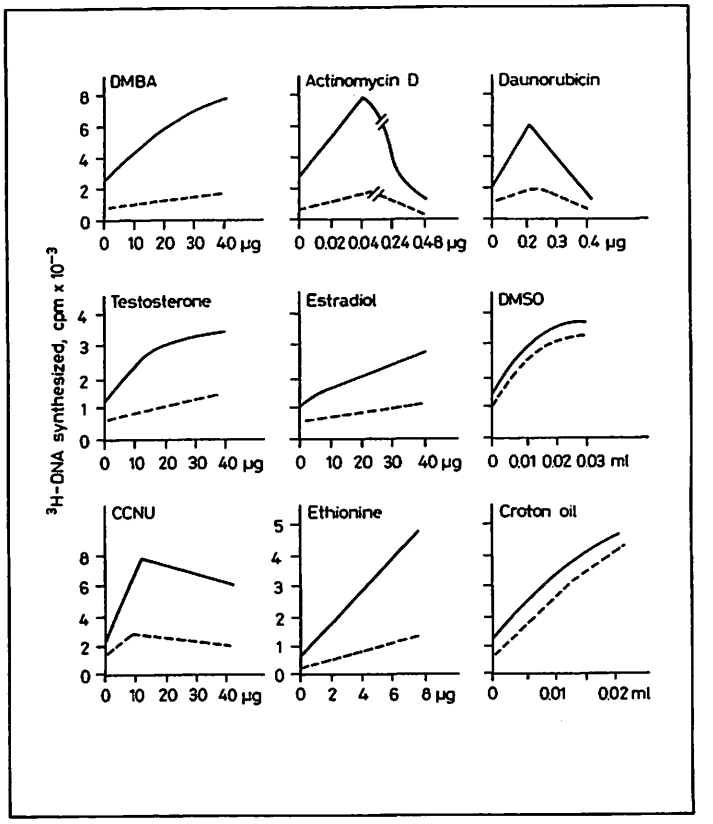


Fig. 1. Cancer and normal in vitro DNA synthesis. Effects of various compounds. Dose-response curves represent cancer DNA synthesis (——) and normal DNA synthesis (– – –). Further explanation in text.

in vitro synthesis. In contrast to carcinogens, drugs and steroids, croton oil and DMSO are almost equally effective on both cancer and normal DNA, as shown in figure 1.

DNA Strand Separation in the Presence of Carcinogens or Other Compounds

In the double DNA helical structure, DNA strands are maintained by hydrogen bonds which can be broken at a relatively high alkaline pH, resulting in an increase in UV absorbance. As shown in figure 2, there is no increase in UV absorbance when cancer DNA or normal DNA is incubated alone at pH 7.60, 7.70 or 7.80. However, beyond pH

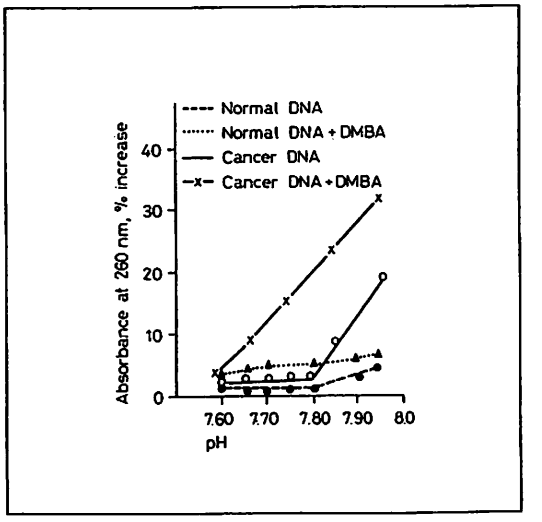


Fig. 2. Cancer and normal DNA strand separation at different pH values. Further explanations in text.

Table I. Comparison of the effects of daunorubicin, dl-ethionine and l-methionine on cancer DNA strand separation

DNA	Absorbance at 260 nm, % increase		
	daunorubucin	l-methionine, 100 µg	dl-ethionine, 100 µg
Breast cancer DNA, 20 µg[1]	21	0	13
Breast cancer DNA, 20 µg[2]	20	0	–
Ehrlich ascites tumor DNA, 20 µg	19	0	17

[1] 4 µg daunorubicin added.
[2] The daunorubicin-treated DNA (footnote 1) was dialyzed (see text) and then reincubated with 4 µg daunorubicin and l-methionine, respectively.

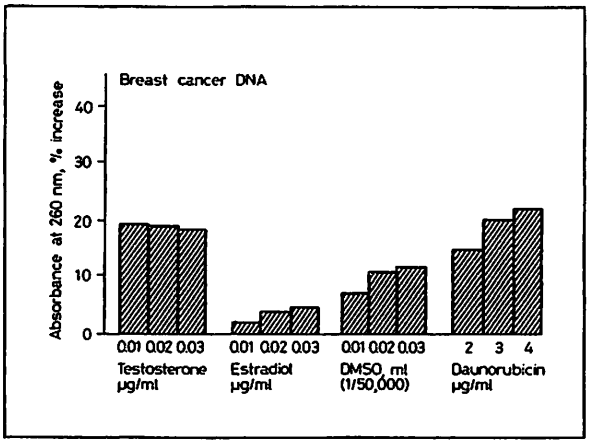
7.85, there is a strong increase in UV absorbance for cancer DNA but not for normal DNA. In the presence of DMBA (10 µg/ml), cancer DNA undergoes complete DNA strand separation while strand separation of normal DNA is weakly increased (fig. 2). Ionic strength plays an important role in this phenomenon (fig. 3). NaCl prevents hydrogen bond breakage by carcinogens.

As shown in figure 4, a progressive increase in UV absorbance of human breast cancer DNA dissolved in 0.01 *M* Tris buffer (pH 7.65) is induced by increasing concentrations of DMBA which, also in the case of Ehrlich ascites tumor DNA, can lead to a 35% hyperchromic effect while, in the case of duck or monkey spleen DNA, i.e. normal DNA, this effect is about 3%.

Ethionine, a potent carcinogen used at rather high concentrations, also induces separation of DNA strands isolated from Ehrlich ascites cells with a maximal increase in hyperchromicity which is of the order of 17% while this increase is about 3% in the cae of duck or monkey spleen DNA; l-methionine has no effect at all in this respect (table I).

Known as anticancer drugs and also known as carcinogens [11, 27, 29], CCNU or actinomycin D induce a strong separation of the strands of cancer DNA and act weakly on normal DNA (fig. 5). DMBA, ethionine, CCNU and actinomycin D separate to a different degree the strands of a given cancer DNA (fig. 4, 5), thus producing results which sugest the existence of different reactive sites on a given cancer DNA. These differences might be responsible for the difference in kinetics and extent of cancer DNA strand separation induced by different substances. When human breast cancer DNA was incubated in the presence of increasing concentrations of either estradiol, DMSO, testosterone or daunorubicin (fig. 6) we observed that estradiol produces a weak increase in UV absorbance while testosterone or daunorubicin is more efficient (with different kinetics) and DMSO is in between (fig. 6). These results suggest that breast cancer DNA possesses different reactive sites whose nature is not yet known.

To verify that a compound such as daunorubicin, which, at a given concentration, in-



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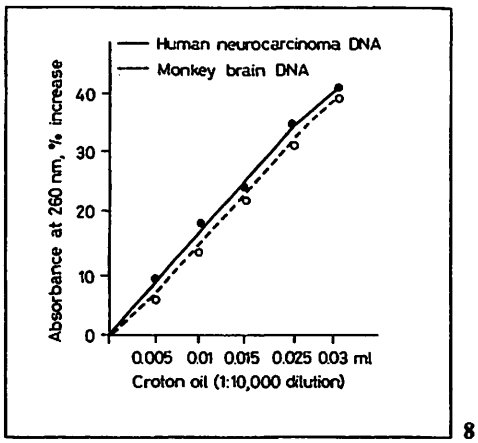

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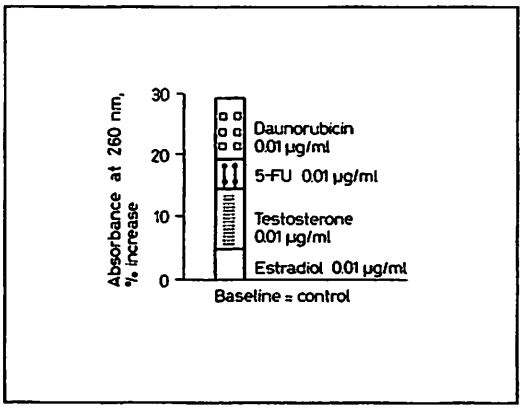

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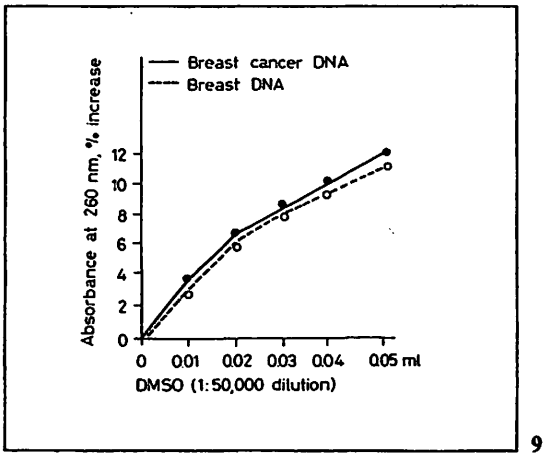

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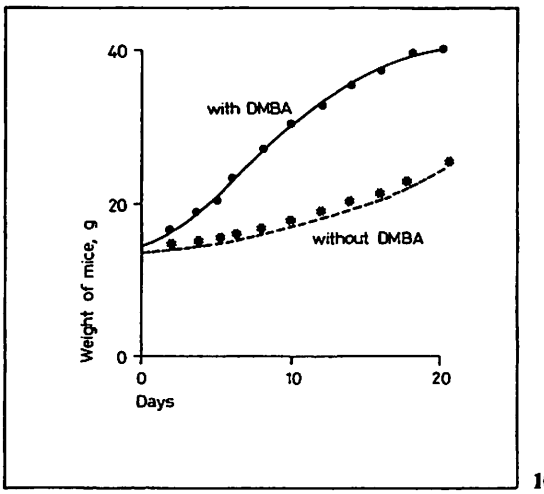
Fig. 6. Human breast cancer DNA strand separation. Effects of estradiol, testosterone, DMSO and daunorubicine. A freshly prepared solution of each compound was used. The DNA samples in the presence of each compound were read against a blank cuvette filled with 10^{-2} *M* Tris-HCl buffer (pH 7.65) containing the compound tested at the indicated concentrations.

Fig. 7. Human neurocarcinoma DNA strand separation. Effects of estradiol, testosterone, 5-FU and daunorubicin. Absorbance at 260 nm of human neurocarcinoma DNA (20 µg), dissolved in 1 ml of 10^{-2} *M* Tris-HCl buffer (pH 7.65).

Fig. 8. Human neurocarcinoma and monkey brain DNA strand separation: effect of croton oil.

Fig. 9. Human breast cancer and normal DNA strand separation: effect of DMSO.

Fig. 10. Stimulation of Ehrlich ascites cells in mice by DMBA. Further explanation in text.



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drugs [31]. These results, and the results which show the additive effects of the above compounds on human neurocarcinoma DNA strand separation, suggest that all cancer DNA must possess some particular sites different for various DNA. The high susceptibility of cancer DNA to the compounds tested indicates that the double helical structure of cancer DNA might be destabilized, for instance by methylated bases whose level could be higher in cancer than in normal DNA. This view is compatible with the observation [13] that the presence of methylated adenine residue in DNA destabilizes the double helices in proportion to the frequency of occurrence of these residues. On the other hand, molecules such as peptides or amino acids bound in a different concentration to cancer and normal DNA, may participate in the stabilization and/or destabilization of the structure of a given DNA.

Of particular interest are the observations made with croton oil [source of phorbol derivatives; 16] and DMSO which, at low concentrations, are capable of enhancing DNA in vitro synthesis and strand separation not only of cancer DNA but also of normal DNA. When DNA strand separation is moderately induced by low doses of croton oil, the addition of DMBA further increases this process. It was demonstrated that the phorbol esters (isolated from croton oil), at high concentrations and in the absence of any other carcinogen, may lead to a low cancer incidence [16] and that the burst of DNA synthesis induced in skin by croton oil is preceded by a smaller and shorter stimulation of RNA and protein synthesis [17], a fact which should be related to the appearance of cancer cells in animals [6, 24]: it has been reported that croton oil is required as a promoter in the carcinogenesis initiated by a carcinogen [25]. Both croton oil derivatives and DMSO are capable of inducing differentiation of some cancer cell lines [14, 18]. Of particular interest is the observation that in human leukemic HL 60 cells, phorbol derivatives, at low concentrations, induced differentiation to macrophages whereas actinomycin D, which is carcinogenic in vitro [2] as well as in vivo [27], induced differentiation to granulocytes [21]. This drug is also capable of inducing neurite formation in mouse neuroblastoma cells [1].

Our in vitro observations on DNA synthesis and strand separation correlate with the multiplication of cancer cells. In fact, DMBA or CCNU, at relatively low concentrations, markedly stimulate the multiplication of Ehrlich ascites tumor cells in mice, while daunorubicin, cyclophosphamide or DMBA stimulate the multiplication of Crown gall tumor cells in plants, only when used at low concentrations: at higher concentrations, they inhibit tumor development [20]. These results might be connected with observations obtained in clinical studies for cancer treatment with chemotherapeutic agents. In order to kill cancer cells, sequential use of different drugs is commonly advised in therapy. It is conceivable that different drugs contribute to separate the strands of cancer DNA above a certain threshold (as we showed in vitro; fig. 7); once this is accomplished, the pathways for synthesis are disconnected and the cell is expected to die.

On the other hand, the relative resistance of DNA from healthy tissues to undergo in vitro DNA strand separation in the presence of carcinogens or other compounds might be connected with the fact that the effect of carcinogens never leads to instant cancer. It was reported that induction of cancer cells probably needs persistent gene activation and that the appearance of cancer cells is an additive

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