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Short Communication

COVALENT BINDING OF DIETHYLSTILBESTROL TO DNA IN RAT AND HAMSTER LIVER AND KIDNEY

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Introduction

Diethylstilbestrol (DES) is a synthetic estrogen still in veterinary practice and used as an anabolic agent in animal feed [1]. In the fifties, high doses of DES were occasionally administered to pregnant women for the preventing of miscarriage. This treatment was associated with an increased risk of vaginal or cervical adenocarcinoma in their female offspring [2]. A very similar transplacental effect was found in various laboratory animal species [3, also refs. therein; 4]. DES was also found to increase the tumor incidence in a number of hormonally dependent sites as well as in lymphoid tissues in mice and in the kidney of hamsters [5].

Most chemical carcinogens exert their activity through covalent interaction of a reactive metabolite with DNA in the target organ [6] and are therefore called genotoxic. Another group of tumor-enhancing agents, viz. cocarcinogens and promoters, do not themselves react with DNA but apparently modulate one or several out of a variety of biochemical and biological steps related to the process of tumor formation. Carcinogenic hormones are widely believed to belong to this latter group of chemicals because of their proliferative effect on target cells. On the other hand, there is evidence that DES can undergo covalent interactions with protein in vivo and with DNA in vitro [7]. Metabolism studies with DES have revealed that monooxygenases convert DES to catechols and to the 3,4-oxide which are chemically reactive [8]. Peroxidases convert DES into β-dienestrol [9], probably via reactive semiquinones and quinones.

Abbreviations · DES, diethylstilbestrol, CBI, Covalent Binding Index.

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It was the aim of this study to determine whether DES is able to bind to DNA in vivo, and if so, to estimate to what extent this genotoxicity could be responsible for the observed carcinogenic potency.

Materials and Methods

All chemicals and reagents were of the highest purity available from Merck, Darmstadt, F.R.G. [Monoethyl-1-^{14}C]DES was purchased from the Radiochemical Centre, Amersham, U.K. in three batches with specific activities of 58, 58 and 60 mCi/mmol, respectively and was used without further purification. The radiochemical purity was >99% as determined by thin-layer chromatography on silica gel in diethyl ether/hexane (1:1). The stock solution was blown dry with nitrogen in order to remove any volatile breakdown fragments and DES was redissolved in ethanol/water and 1,2-propanediol/water (1:1), for oral and subcutaneous administration, respectively.

Young adult female rats (Sprague—Dawley-derived ZUR:SIV-Z; 214—228 g) and male golden Syrian hamsters (ZUR:LAK-Z; 106—111 g) were used. Eight hours after the administration of 0.5 ml of the DES solution (oral for rats, s.c. for rats and hamsters) liver and kidney were excised and DNA was isolated immediately and prepared for liquid scintillation counting as described [10]. In the first experiment with rats, DNA of spleen and lung were also studied.

In order to check whether the method used for the isolation of DNA removes non-covalently bound DES, a control liver homogenate was incubated with 8 μCi [^{14}C]DES for 4 h at room temperature. 5.8 mg DNA isolated thereafter did not contain any significant radioactivity at a limit of detection of 1.5 dpm.

Results

The specific activities of the DNA's isolated after treatment of the animals with [^{14}C]DES were transformed to the units of a DNA binding in 'molecules of DES bound per 10^6 DNA nucleotides', and this damage was normalized to a theoretical dose of 1 mmol DES administered per kg body weight. This conversion of the raw data to a Covalent Binding Index (CBI; see also footnote to Table I for definition) will allow a quantitative comparison of the DNA-binding capacities of different carcinogens.

The data given in Table I show that after oral administration of DES to rats a mean CBI of 0.5 was determined for liver DNA. No radioactivity was measurable in the DNA of kidney, spleen or lung. After subcutaneous administration, the CBI for liver DNA was slightly higher, but, again, no radioactivity could be detected in the kidney (Table II). Subcutaneous administration to the hamster resulted in a higher binding as compared with the rat (Table III). The difference was of a factor of about ten in the liver and of at least five in the kidney.

The specific activity measured in the DNA was extremely low and allowed no further proof for true covalent binding, for instance by degradation of

TABLE I

BINDING OF DES TO DNA OF FEMALE RAT LIVER, KIDNEY, SPLEEN, AND LUNG, 8 H AFTER ORAL ADMINISTRATION OF THE ^{14}C-LABELLED DRUG

Animal wt (g)	215				214			
Chemical dose (mg/kg body wt.)	2.2				2.6			
Radioactivity dose (dpm/kg)	$1.03 \cdot 10^9$				$1.25 \cdot 10^9$			
	Liver	Kidney	Spleen	Lung	Liver	Kidney	Spleen	Lung
Amount of DNA in scintillation vial (mg)	10.8	3.25	3.46	3.56	12.7	1.98	2.96	3.14
Gross activity (±1.2 cpm)	26.5	22.8	22.9	22 8	41.9	23.1	23.2	22 9
Background DNA activity (±1.2 cpm)	20.8	22.5	22.5	22.5	20.8	22.5	22.5	22.5
Specific activity (dpm/mg DNA)	1 0	<0 7	<0.7	<0.7	3.0	<1.4	<1 0	<0 8
CBI[a]	0 3	<0.2	<0 2	<0 2	0.7	<0 3	<0.2	<0.2
Mean CBI for liver DNA	0 5							

[a] The Covalent Binding Index [CBI, 6] is defined as

$$CBI = \frac{\mu\text{mol of chemical bound/mol DNA nucleotide}}{\text{mmol of chemical applied/kg body wt}} = \frac{\text{dpm/mg DNA}}{\text{dpm/kg body wt}} \times 3\ 09 \cdot 10^8$$

TABLE II

BINDING OF DES TO DNA OF FEMALE RAT LIVER AND KIDNEY, 8 H AFTER SUBCUTANEOUS ADMINISTRATION OF THE ^{14}C-LABELLED DRUG

Animal wt. (g)	228		224	
Chemical dose (mg/kg body wt)	1.8		2 0	
Radioactivity dose (dpm/kg)	9 16 · 10^8		9 97 · 10^8	
	Liver	Kidney	Liver	Kidney
Amount of DNA in scintillation vial (mg)	7.31	4 34	6 26	2 88
Gross activity (±1.2 cpm)	27 8	17 9	27 8	17 4
Background DNA activity (cpm)		16 4 ± 1 2		
Specific activity (dpm/mg DNA)	1.9	<0 5	2 2	<0 7
CBI[a]	0 6	<0 2	0 7	<0 2
Mean CBI for liver DNA	0 6			

[a] See footnote to Table I.

TABLE III

BINDING OF DES TO DNA OF MALE HAMSTER LIVER AND KIDNEY, 8 H AFTER SUBCUTANEOUS ADMINISTRATION OF THE ^{14}C-LABELLED DRUG

Animal wt. (g)	106		111	
Chemical dose (mg/kg body wt.)	6 9		5 0	
Radioactivity dose (dpm/kg)	3 30 · 10^9		2 36 · 10^9	
	Liver	Kidney	Liver	Kidney
Amount of DNA in scintillation vial (mg)	3 13	2 06	2 79	2 41
Gross activity (±1 2 cpm)	149	43	74	25
Background DNA activity (cpm)		17 0 ± 1 2		
Specific activity (dpm/mg DNA)	49 5	15.0	24 0	3 7
CBI[a]	4 6	1 4	5 6	0 5
Mean CBI for liver/kidney DNA	5	1		

[a] See footnote to Table I

the DNA and chromatography of the resultant nucleosides. Non-covalent binding of DES to DNA can, however, be excluded on the basis of the control experiment which showed that unbound DES is completely removed during the purification of the DNA. Biosynthetic incorporation is another possibility to produce radiolabelled DNA. Control experiments (data not shown) with [^{14}C]methanol showed that incorporation of carbon-1-pool precursors into DNA gives rise to an apparent binding index for rat liver DNA of about 170, 12 h after oral administration. As little as 0.3% of the DES dose, if degraded to carbon-1 fragments, could already lead to the observed radioactivity in rat liver DNA. Two main arguments can be put forward to make this possibility unlikely: (i) a metabolic degradation of DES leading to the loss of the label used has never been observed [7] and can, therefore, almost be excluded; (ii) the biosynthetic incorporation of radiolabel from [^{14}C]methanol into the DNA in the kidney was found to be slightly higher than in the liver (control data; not shown). This is in contrast to the respective radioactivities measured after treatment with DES.

In addition, DNA binding of DES metabolites has been detected in vitro [9,11], and it is not surprising that, qualitatively, the same can happen in vivo. We therefore believe that the radioactivity associated with the DNA reflects true covalent binding.

The binding level of DES to DNA was about one order of magnitude higher in the hamster than in the rat. For the kidney, this fact could be explained by the presence of peroxidase, an enzyme which is lacking in mice and rats [12]. The hamster kidney might, therefore, represent a special case with respect to the activation of DES to DNA-binding metabolites. In the liver, other enzymatic activities must, however, be responsible for this activation because the liver of neither rodent species did contain detectable peroxidase activity [12], and still the CBI in hamster liver was higher than in the liver of the rat.

Discussion

CBI-values for about 80 compounds have been compiled in a recent review [6] from the literature and from data collected in this laboratory. It could be shown that CBI reflect the genotoxic carcinogenic potency of a chemical in long-term bioassays. CBIs for liver DNA of the order of 10^3—10^4 are found for potent hepatocarcinogens, of 10^2 for moderate and around 10 for weak hepatocarcinogens [6]. If non-hepatocarcinogens are also included, the correlation still holds [13], and a very recent analysis of the data also revealed that the CBI for liver DNA will also allow the classification of a genotoxic carcinogen into categories of different potencies, even if the target organ for the tumor incidence is not the liver [14]. This is in agreement with the empirical finding that typical genotoxic carcinogens always exhibit high CBIs in the liver, even if the liver is not a high-risk organ in terms of the carcinogenic effect.

If a compound exhibits a low CBI of around 1, but has been shown to be a moderate to strong carcinogen, biological effects in addition to geno-

toxicity, such as cytotoxicity, hormonal or promoting activity, can always be found, and the over-all carcinogenicity must be based upon the sum of a variety of effects. Such seems to be the case also for DES: TD_{50}-values* of about 0.1 μmol/kg/day were estimated for the mouse mammary gland [15,16] and an about 100% kidney tumor incidence in male hamsters was obtained with effective daily doses of DES ranging between 0.3 and 3 μmol/kg/day, depending on the mode of administration [5,17]. By using the correlation of CBI vs. TD_{50} [13], CBI-values of the order of 10^2—10^3 would be expected in the organs at risk if the mode of carcinogenic action of DES was purely by DNA binding. The actually measured CBI-value of 1 for hamster kidney DNA (or an even lower CBI-value, if not all radioactivity was due to covalent binding) is two to three orders of magnitude lower than would be expected on the basis of long-term carcinogenicity data [13].

The above quantitative analysis used for evaluation of the mechanism of carcinogenic action of a test compound is obviously not complemented by an equal precision regarding the estimation of carcinogenic potencies. Nevertheless, we believe that the multistep process of chemical carcinogenesis must ultimately be dissected if short-term test and animal data are to be extrapolated to man on a scientific basis.

The lack of a correlation of the small genotoxicity demonstrated here with the carcinogenicity found in long-term bioassays is in agreement with the negative findings for a mutagenicity of DES in *Salmonella* [18], V79 cells [19] or Syrian hamster embryo cells [20]. DES has also been found to induce neoplastic transformation of Syrian hamster embryo cells *without* inducing mutations in genes with testable function [20]. Some type of DNA interaction has, however, been detected earlier [8], e.g., DES has been found to induce unscheduled DNA synthesis in HeLa cells in the presence of rat liver microsomes [21]. DES also decreases the fidelity of DNA synthesis in vitro [22] and induces sister chromatid exchanges in cultured human fibroblasts [23].

The above-mentioned in vitro tests can, however, not be used for a *quantitative* extrapolation to an intact mammalian organism because of the well-documented distortion of the enzymatic activation and inactivation pathways governing the concentration of the ultimate carcinogens. The in vivo data presented here indicate that mechanisms other than direct genotoxicity may be more important for the carcinogenicity of DES. Most obviously, the hormonal activity of DES might be responsible for most of its carcinogenic activity, and it is conceivable that also the transplacental effects are based upon this type of activity. These hypotheses are substantiated by the finding that antiestrogens largely inhibit tumor formation by DES and other estrogens [24,25]. Also, α-fetoprotein, the major estrogen-binding component in the rodent fetus [26] traps DES to a much lesser extent than steroid estrogens so that at equimolar doses a higher fraction

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*A TD_{50} is that daily dose of chemical which induces a 50% tumor incidence in animals treated for life

of DES can accumulate in the fetal genital tract and disturb development much more efficiently than can steroid estrogens.

In conclusion, DES was shown to exhibit a very low degree of covalent binding to DNA of rat liver and hamster liver and kidney. Expressed per unit dose as a CBI, this genotoxic activity is, however, so low for DES that mechanisms other than DNA modification (probably hormone-mediated mechanisms, such as an increased rate of cell proliferation) are considered more likely to be responsible for the carcinogenic effect of DES observed in animals.

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