Role of Metabolism and DNA Adduct Formation in the Induction of Sister Chromatid Exchanges in Human Lymphocytes by Diethylstilbestrol

Karsten Lundgren, Kurt Randerath, and Richard B. Everson

Epidemiology Branch, Biometry and Risk Assessment Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 [K. L., R. B. E.], and Department of Pharmacology, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77030 [K. R.]

ABSTRACT

Recent studies have shown that diethylstilbestrol (DES) induces sister chromatid exchanges (SCEs) in lymphocytes from pregnant and premenopausal women but has only a slight effect on lymphocytes from postmenopausal women or men. In this study blood specimens from premenopausal women were used to define the role of different metabolic pathways on DES-induced formation of SCEs and to determine whether conditions resulting in induction of SCEs also induced detectable levels of DNA adducts. Exposure of lymphocytes in vitro to 0–40 μM DES induced a concentration-dependent increase in SCEs. Addition of indomethacin to the cultures partially abolished DES-induced SCEs, suggesting involvement of prostaglandin synthetase in the formation of specific DES metabolites that cause SCEs. α-Naphthoflavone, an inhibitor of cytochrome P-450 monoxygenases, had no effect on DES-induced SCEs. Cells exposed to DES at doses sufficient to cause large increases in SCE induction did not have adducts detectable by a 3P-postlabeling assay capable of revealing adducts at a level of 1 adduct/10⁶ normal nucleotides.

INTRODUCTION

Transplacental human exposure to DES causes clear cell adenocarcinoma of the vagina in women (1, 2) and nonmalignant alterations in the reproductive tracts of male and female progeny (3–5). The mechanism by which DES causes cancer and malformations is unknown, but both the estrogenic properties and ability of DES to alter genetic material have been implicated.

Recent studies of the effects of DES on genetic material suggest that its actions vary in different experimental systems. DES induced chromatid-type chromosomal aberrations but not SCEs in vivo in mouse bone marrow (6); female mice were more susceptible than male mice. SCEs were induced in cultured metabolically active hepatoma cells (7) and fibroblasts (8), but not in other fibroblast systems (7, 9, 10). Using lymphocytes from premenopausal and pregnant women, Hill and Wolff (11, 12) demonstrated induction of SCEs by in vitro exposure to DES. Exposure of lymphocytes from men or postmenopausal women to similar doses of DES had only a slight effect on SCE formation, and exposure of lymphocytes from premenopausal women to the natural estrogens estradiol and estradiol did not induce SCEs. Using a 3P-postlabeling assay, Liehr et al. (13) recently demonstrated that DES induces DNA adducts in Syrian hamsters through an indirect mechanism; adduct formation was specific for the kidney, the target organ for DES carcinogenesis in the Syrian hamster.

Since SCEs may result from direct interaction of a reactive metabolite of a chemical with DNA, differences in the effects of DES in different test systems may be due to capabilities of the cells under study to metabolically activate DES. There is evidence to suggest that either the PGS system or the cytochrome P-450 monoxygenase system could be involved. In Syrian hamster fibroblasts in culture, the PGS system metabolized DES to reactive stilbenes or Z,Z-dienestrol (14–17). Metabolism by this pathway can be specifically inhibited by indomethacin (18). Possible involvement of P-450 monoxygenase pathways was suggested by experiments showing a large decrease in the number of renal tumors induced in Syrian hamster kidney when ANF, a drug that inhibits the P-450 enzymes by competitive binding, was included in the diet (19) and by studies showing that metabolic activation by hepatic microsomes mediated DNA binding of DES (20).

The differential DES sensitivity of cells from premenopausal and postmenopausal women implies extraordinary target specificity. Hypothesizing that such specificity might be mediated by patterns of DES metabolism in lymphocyte cultures, we used the inhibitory properties of indomethacin and ANF to investigate whether the PGS or the P-450 metabolic pathways could be implicated in the in vitro formation of DES-induced SCEs in lymphocytes of premenopausal women. In addition, since sister chromatid exchanges could result from chemical addition products on DNA (DNA adducts), we investigated whether in vitro exposure of lymphocytes from premenopausal women to concentrations of DES shown to cause induction of SCEs resulted in the formation of DNA adducts detectable by a 3P-postlabeling assay (13, 21, 22). This recently developed assay is capable of detecting very low levels of DNA adducts formed by aromatic chemicals.

MATERIALS AND METHODS

Heparinized blood was obtained by venipuncture from healthy, non-smoking, premenopausal women, who reported no recent exposure to prescription medications. Whole blood (0.5 ml) was added to RPMI 1640 (Gibco) culture medium supplemented with fetal calf serum (1.5 ml; Gibco), PHA (2%; Gibco), penicillin plus streptomycin (100 units + 100 μg/ml; Gibco), 1-glutamine (2 mM; Sigma), and 5-bromo-2-deoxyuridine (20 μM; Sigma) to yield a total volume of 10 ml. DES, indomethacin, or ANF (all Sigma) was dissolved in dimethyl sulfoxide in appropriate concentrations and added to the culture media as 20-μl aliquots where indicated. Equivalent amounts of DMSO were added to the control cultures. Samples were harvested 72 h following a 2-h incubation with Colcemid (0.08 μg/ml; Gibco). Processing of samples and slides for analysis of SCEs was carried out as described previously (23). SCEs were evaluated under the microscope at ×1260 in metaphase spreads having at least 44 chromosomes. Except in a few instances where inadequate numbers of scorable metaphases were available, for each individual 50 cells were scored from untreated cultures and 25 cells were scored for each test condition.

For the DNA adduct studies, mononuclear peripheral blood cells were isolated in Ficoll-Paque (Pharmacia) gradients and washed 3 times in RPMI 1640. Cells were incubated at a concentration of 1 × 10⁶ cells/ml in RPMI 1640 supplemented with fetal calf serum (15%) and 1% streptomycin–penicillin–ampicillin. Exposure of lymphocytes to DES was for 24 h (up to 40 μM) in RPMI 1640. Cells were then harvested and 3P-postlabeling was performed as described previously (13). Quantitation of DNA adducts was determined by liquid scintillation counting of ρ-3P-phosphoethanolamine, ρ-3P-phosphoethanolamide, ρ-3P-phosphoethanolamine, ρ-3P-phosphoethanolamide.
1-glutamine (2 mM) with or without 2% PHA. DES, dissolved in DMSO at concentrations of 40 or 200 μM, was added to the cultures in 20-μl aliquots prior to incubation. Twenty μl of DMSO were added to the control cultures. Cultures were incubated either with PHA for 72 h or without PHA for 24 h. At the end of the culture period, the viability of the cells was assessed by trypan blue exclusion; viability was always greater than 95%. DNA was isolated from these cells and assayed for adducts using methods described by Randerath et al. (22). This assay is performed by digesting the DNA with endonucleases to deoxyribonucleoside 3'-monophosphates, labeling the monophosphates with [γ-32P]-ATP, and separating the 32P-labeled normal and adducted monophosphates by thin layer chromatography. Adducts are then detected by formation of spots on a screen-intensified autoradiogram of the thin layer chromatograms. In order to detect the low levels of adducts, the conditions used for [γ-32P]-ATP labeling in this study were modified as described by Randerath et al. (22) to increase the relative yield of adducted nucleotides by using a large excess of deoxyribonucleoside 3'-monophosphates and high specific activity of [γ-32P]ATP labeling. Labeling of certain adducts is increased because in many instances the T4 polynucleotide kinase labels the aromatic deoxyribonucleoside 3'-monophosphate adducts at a higher rate than unadducted deoxyribonucleoside 3'-monophosphates (22). This preferential labeling of adducts usually results in a 20- to 100-fold intensification, increasing the sensitivity of the assay.

RESULTS

DES caused a concentration-dependent increase in the frequency of SCEs (Table 1). The mean observed SCE frequencies were 8.1, 8.8, 9.4, and 10.0 at DES concentrations of 0, 10, 20, and 40 μM, respectively. The response of different individuals appeared to be similar for most subjects; the exceptions were a decrease in SCE levels at the highest dose for the only assay for one subject (subject 5) and one of three replicate assays for a second subject (subject 11). Cells from some of the assays where the dose-response curves declined had unusually contracted chromosomes, making scoring difficult. At a concentration of 80 μM, DES was highly cytostatic, resulting in an absence of second division metaphases after 72 h incubation so that SCE frequencies could not be determined at this concentration. Among individuals, variability in the frequencies of SCEs as measured by the coefficient of variation was not increased by exposure to DES.

The data shown in Table 1 were further analyzed by linear regression techniques using a computer program for a generalized linear model (Statistical Analysis Systems, Inc.). If individual differences in the baseline SCE frequencies are ignored, the data for all subjects fit the equation SCE = 8.20 + 0.48X, where X is the DES dose (in μM × 10^3). For this model R^2 = 0.22 and P < 0.0004. The addition of terms for individual differences in baseline SCE frequencies among study subjects improves the predictive value of the model significantly (P < 0.0001), giving the model R^2 = 0.77 and P < 0.0001. This improvement implies that there was significant variability in the base line frequencies of SCEs among subjects in this study. The further addition of interaction terms assessing differences in the dose response for different individuals did not significantly improve the model (P = 0.14). Lack of improvement with the addition of this term to the model indicates that the variability in slopes of the DES dose-response curves among different subjects was not statistically significant in this study.

Table 2 shows the effect of indomethacin on the frequency of base line and DES-induced SCEs. An indomethacin concentration of 50 μM was selected based on preliminary experiments (data not shown) indicating no effect of indomethacin at lower concentrations and cytotoxicity at higher concentrations. Indomethacin by itself had no significant effect on the frequency of SCEs but was able to significantly limit the effects of DES. Preliminary experiments with ANF indicated that it induced SCEs in lymphocytes from smokers to a much greater extent than from nonsmokers. This finding was further investigated elsewhere (23) and only nonsmokers are reported in this study.

Fig. 1 shows results obtained by coincubation of lymphocytes with DES and ANF. Analysis of these experiments by linear regression indicated that DES alone induced a dose-related increase in the frequencies of SCEs (P = 0.02) and ANF alone at a concentration of 40 μM significantly increased levels of SCEs (P < 0.01). Combination of ANF and DES caused the largest increase in SCEs. Statistical evaluation indicated that these increases were best explained by a simple additive rather than a synergistic model [the estimated parameter for interaction between ANF and DES in a multivariable model of SCE values was not significant by an F test (P = 0.20)]. Thus, the
increases seen with the combination of DES and ANF appeared to be related to the individual effects of DES and ANF rather than an effect of ANF on metabolism of DES. Differences between the mean SCE frequencies of cultures with no DES or ANF and cultures with 20 μM DES plus 40 μM ANF were 2.7, 3.3, 3.9, 4.5, 4.6, and 4.7 SCEs for the six subjects studied, while control cultures and those treated with 40 μM DES plus 40 μM ANF differed by 3.6, 3.5, 7.1, 5.2, 5.4, and 2.2 SCEs for the same six subjects, respectively. There are not sufficient data to allow statistical analysis of individual differences in response to combined ANF and DES.

Autograms of thin layer chromatograms from the 32P-postlabeling assays were identical for control lymphocytes and lymphocytes incubated for 24 or 72 h with various concentrations of DES. Thus, there was no indication of the presence of DNA adducts associated with DES.

**DISCUSSION**

This report confirms that *in vitro* exposure to DES (0 to 40 μM) induces a concentration-dependent increase in SCEs in primary cultures of human lymphocytes from premenopausal women. Indomethacin was partially able to abolish DES-induced SCEs. Indomethacin is known to block the formation of prostaglandin G2 and thereby the peroxidative mediated metabolism of DES. Our results suggest that PGS is involved in the formation of DES metabolites causing SCEs. However, the complete inhibition of DES-induced SCEs indicates that other mechanisms might be involved as well or that complete inhibition of PGS did not take place with the concentration of indomethacin used. The latter possibility seems unlikely, however, since Darrow and Tomar (24) showed complete inhibition of PGS activity in mixed lymphocyte reactions with indomethacin concentrations 3 times less ours. In contrast to our results, Mehnert *et al.* (10) reported that oxidative metabolism by coincubation of lymphocytes from premenopausal women, horseradish peroxidase, and peroxide with increasing doses of DES did not result in a dose-dependent association between DES concentration and SCE formation. Small increases were noted for cells receiving peroxidase and peroxide compared with cultures receiving only DES. Their experimental approach, however, differed from ours in that exposure to DES and peroxides took place for only 2 h and only two pregnant women were studied. Also, experiments by Hill and Wolff (12) showed that coincubation of DES with lymphocytes from a male and lymphocytes from a female resulted in SCE formation only in the lymphocytes from the female, suggesting that metabolites causing the increase in SCEs are not diffusible through tissue culture media. This being the case, exogenous activating systems would not be expected to have effects comparable to activation by intracellular processes.

ANF was added to the cultures to test if SCE induction was mediated by metabolism of DES through the P-450 monoxygenase system. In agreement with previously published results (23), ANF was shown to be a weak inducer of SCEs. However, ANF did not inhibit DES-induced SCEs. These data disagree with results obtained for human fibroblast cultures demonstrating that ANF totally inhibited DES-induced SCEs (8). It is not clear whether this discrepancy is due to metabolic differences between the two cell types or the lower DES concentration used by Rudiger *et al.* (8) compared with that used in this study.

Using DES concentrations up to levels shown to induce SCEs and impair cell replication severely, no DES adducts were detected by the 32P-postlabeling assay. The methods used should have detected a wide range of relatively large aromatic addition products at levels of 1 adduct in 10⁶ normal nucleotides, such as modifications that might be expected if a metabolite of DES formed adducts. The chromatographic methods used would not be able to detect small addition products such as methyl or ethyl groups (21). Although unstable adducts or adducts not detectable by this procedure may have been formed, these results suggest that covalent DNA alteration by DES is not likely to be responsible for the formation of SCEs by DES.

Less than 1.4 in 1000 females prenatally exposed to DES develop vaginal adenocarcinoma (25), suggesting that susceptibility factors may be important determinants of individual risk. Involvement of peroxidative metabolism in the induction of SCEs by DES raises the possibility that levels of PGS activity or levels of related enzymes may be one such susceptibility factor. That possibility and others could be examined by using laboratory approaches as described in this study to analyze specimens from DES-exposed women who had varied clinical effects from DES, such as no apparent abnormalities, genital tract malformations, or malignancy. As presented in Table 1 and the statistical analysis of that table included under "Results," variability in the response to DES was not statistically significant among the 11 individual women studied here. However, for this study women were not selected because of DES-related disease and their number is too small to detect a rare susceptibility factor in the general population. Also, while differences described here such as the 24% increase in the frequency of SCEs induced by DES appear relatively modest, studies using SCEs have relatively good statistical power; according to one estimate, as few as 5 individuals per group are sufficient to distinguish a 24% increase if 50 metaphases per subject were scored (26). Analysis of SCEs should have reasonably good statistical power for detecting variability in SCE response among different women. Thus, studies applying the laboratory approaches described here to relatively small groups...
of subjects could provide leads to susceptibility factors for DES
 teratogenesis or carcinogenesis in humans.

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