ABSTRACT

Despite an increasing incidence of human breast cancer, its etiology remains unknown. Since some environmental chemicals are stored in human breast fat and are rodent mammary carcinogens, determining the genotoxic potential of environmental agents in this key target tissue is important. An assay was developed for detecting genotoxic activity, as unscheduled DNA synthesis (UDS), induced by chemicals and UV radiation in early passage cultures of normal human mammary epithelial cells (HMEC) derived from 5 different women. In order to measure UDS in culture, reduction in the percentage of cells in S-phase was accomplished either by depriving the cells of epidermal growth factor and bovine pituitary extract or by contact inhibition of growth. Cultures were incubated with test chemicals for 24 h in the presence of [3H]thymidine. UDS was quantitated autoradiographically as net grains per cell. A positive response was observed with UV radiation, benzo(a)pyrene, aflatoxin B1, ethylmethanesulfonate, 1,6-dinitropyrene, 2-acetylaminofluorene, and tobacco smoke condensate but not 7,12-dimethylbenz(a)anthracene or 2,3,7,8-tetrachlorodibenzo-p-dioxin. These results demonstrate that HMEC from all 5 women examined have the ability to metabolize a variety of environmental chemicals to DNA-reactive forms. Furthermore, some chemicals known either to cause mammary cancer in rodents or to be contaminants in human breast tissue are genotoxic in HMEC. A positive response in passage 9 cultures was observed only with direct acting agents, suggesting that HMEC may lose their metabolic capabilities in longer-term cultures. The HMEC UDS assay may be used to address the role of environmental agents in human breast cancer by determining whether chemicals are DNA reactive or metabolized to DNA reactive species in this critical target tissue.

INTRODUCTION

Breast cancer is the most common cancer in American women, resulting in a mortality second only to the mortality caused by cigarette-induced lung cancer (1). Further, the incidence of breast cancer is increasing at a steady rate (1). Despite its prevalence, the etiology of human breast cancer is poorly understood. While many environmental variables such as diet may modulate the promotion and/or progression of breast cancer, the only environmental agent that has been proven to induce breast cancer in women is ionizing radiation (2, 3). While no clear epidemiological link between environmental chemicals and human breast cancer has been established, it has been suggested that passive smoking may increase the risk of breast cancer (4). The ability of environmental xenobiotics such as PAHs to induce mammary cancer in rodents is, however, well documented (5, 6). Furthermore, ubiquitous environmental pollutants such as PAH can be stored in human breast fat (7), suggesting that human breast tissue might be exposed to potential carcinogens. Thus, it is important to determine if the human breast can metabolically activate carcinogens and if it may be a target for neoplastic transformation by environmental agents. The question of the relevance of rodent studies to human carcinogenesis is of major concern. One approach to this question is to develop in vitro models for key biological activities of environmental chemicals using human cells.

Numerous genotoxicity assays successfully identify the potential carcinogenic activity of chemical agents in the primary biological activity of which is alteration of the DNA (8, 9). One widely used genotoxicity assay is the measurement of chemically induced DNA repair as a means of assessing the ability of a test agent to reach and alter the DNA. [3H]Thymidine incorporated into the DNA of nondividing cells during excision repair is used to quantitate DNA repair and has been termed UDS (10). DNA repair assays have been described for a variety of rodent and human cell types (10–18). Since species and tissue specificities are observed in chemical carcinogenesis (19), it is important to establish predictive tests in target cells. Therefore, an assay was developed to measure chemically induced DNA repair as UDS in normal HMEC. This assay provides a qualitative indication of potential risk rather than a quantitative risk assessment.

MATERIALS AND METHODS

Cells. Normal HMEC were derived from residual surgical material from reduction mammoplasties of five healthy women 19 to 22 years of age (Cases B402, B499, B708, 3014, and 3085). Passage 9 normal HMEC (Case 184) were a generous gift from Dr. Martha Stamper and were derived from a 21-year-old woman.

Procedures for the isolation and cultivation of HMEC have been previously described (20, 21). Cells were maintained in 100-mm plastic tissue culture dishes (Lux; Miles Scientific, Naperville, IL) in serum-free MCDB 170 medium (Clonetics, San Diego, CA) previously described (22). The supplements in this medium are both bovine pituitary extract (70 μg/ml of total protein; Hammond Cell Technologies, Palo Alto, CA), 1 mM isoproterenol (Sigma Chemical Co., St. Louis, MO), 5 μg/ml of transferrin (Sigma), 5 ng/ml of epidermal growth factor (Collaborative Research, Waltham, MA), 5 ng/ml of insulin (Collaborative Research), 0.5 μg/ml of hydrocortisone (Sigma), 0.33 mg/ml of glucose (Sigma), and 620 μg/ml of gentamicin (United States Biochemical Corp., Cleveland, OH). For growth factor-deprived cultures, epidermal growth factor and pituitary extract were omitted from the medium. Cultures were incubated in a 37°C incubator with 5% CO2 and 95% relative humidity.

Chemicals. Concentrated stock solutions of test chemicals were prepared in DMSO or MCDB 170 medium, depending on solubility. These were added to MCDB 170 medium containing 10 μCi/ml [3H]thymidine (specific activity, 50–90 Ci/mmol; Du Pont, Wilmington, DE). The concentration of DMSO never exceeded 0.25% (v/v). Lower concentrations of chemicals were prepared by serial dilution in MCDB 170 medium containing [3H]thymidine. 2-AAF (>95%), AFB1 (A grade), BP (98%), dimethyltioarsine (>99%), and ethylmethanesulfonate (99%) were purchased from Sigma. DMBA (>98%) and 1,6-DNP (98%) were purchased from Aldrich (Milwaukee, WI). TSC (2R1
reference cigarette) was obtained from the Tobacco and Health Research Institute, University of Kentucky. TCDD (KOR Isotopes, Inc., Cambridge, MA) was provided by Dr. William F. Greenlee, Chemical Industry Institute of Toxicology.

UDS Assay. The in vitro UDS assay described for rat hepatocytes (12) was modified for HMEC. Secondary cultures of HMEC (Cases B402, B499, B708, 3014, and 3085) and passage 9 cultures of Case B18 were split, approximately 10^5 cells/well into 4-well chamber slides (Nunc, Inc., Naperville, IL). Cells were fed with MCDB 170 medium twice weekly until confluency was reached or refed with growth factor-deprived MCDB 170 medium after approximately 1 week. Five days after reaching confluence or after being growth factor-deprived, cells were treated with test agents.

HMEC were incubated in MCDB 170 medium containing 1% fetal bovine serum (Irvine Scientific, Palo Alto, CA), 10 μCi/ml [3H]thymidine, and test chemical for 24 h at 37°C. Controls received medium alone or medium plus no more than 0.25% DMSO. For exposure to UV radiation, medium was aspirated from the cultures, and uncovered culture slides were exposed to UV radiation (254 nm peak wavelength, germicidal lamp) using the Stratalinker UV Crosslinker 1800 (Stratagene, La Jolla, CA). The incident dose rate was 28.7 J/m²/s of UV light as measured with a Spectroline DMX-254X radiometer. Immediately after exposure, MCDB 170 medium containing 1% fetal bovine serum and 10 μCi/ml [3H]thymidine was added to the slides for a 24-h incubation at 37°C.

Following the 24-h incubation period, cultures were washed with MCDB 170 medium containing 1% fetal bovine serum and 25 mM unlabelled thymidine. Medium was replaced with a 1% sodium citrate solution for 10 min to swell the nuclei. For fixation, the sodium citrate solution was replaced with acetic acid:absolute ethanol (1:3) for 10 min. This step was repeated twice more, followed by washing 3 times with distilled water. The plastic wells were then removed from the glass slides, which were allowed to air-dry overnight.

For autoradiography, slides were dipped in undiluted NTB-2 photographic emulsion (Kodak, Rochester, NY), air-dried overnight at room temperature, and exposed for 12 days at −20°C. Slides were thawed at room temperature for at least 3 h, developed at 15°C for 4 min in Kodak D-19 developer, rinsed in 15°C water, and fixed in Kodak Fixer for 3 min with agitation about every 60 s. Slides were then washed in gently running water for 25 min, stained with hematoxylin and eosin while still wet, allowed to air-dry and coverslipped.

Evaluation of Results. NG were defined as silver grains over the nucleus minus the grains over an equal-sized area in the cytoplasm adjacent to the nucleus, and was quantitated with an Optomax 40–10 image analyzer interfaced to a Zeiss Universal microscope. Data were fed directly into a VAX 6210 computer via an ARTEK BCD-RS232 Omni-interface. Counting was done under oil immersion with a 100x objective. Any individual cell with ≥6 NG was considered in repair. The population average NG for 25 to 50 cells was calculated for each of 2 slides per treatment group. The percentage of cells in repair was also calculated for each slide as an indication of the extent of the UDS response in the population.

The unpaired t test for the equality of two means was used to compare NG between control and treated cultures. The χ² test was used to test for significant differences in percentage of cells in repair between control and treated cultures. A response was judged positive for those agents that produced a significant increase in NG and/or percentage in repair compared to controls at a level of P ≤ 0.05.

RESULTS

For the conditions described here, NG ≥6 was chosen for determining whether an individual cell was positive for UDS or "in repair." This value was selected based on a histogram of the percentage of cells in control cultures versus NG (Fig. 1) which revealed that only approximately 4% of control cells were classified as in repair using a NG cutoff of 6. Individual cases were selected to illustrate different profiles that might be observed when comparing to the historical control profile. An example of the type of shift in the histogram for an agent that produced a weak response is also presented in Fig. 1. While the population average for these cells exposed to TSC was only 4 NG, percentage in repair was 33%, which was significantly greater than controls. The histogram shifted even further to the right for cells exposed to AFB1 (Fig. 1), an agent which produced a strong positive response as determined by NG (population average, 18 NG; percentage in repair, 72%).

Photomicrographs of control and treated secondary cultures of HMEC are shown in Fig. 2. Average control values ranged from −2.89 to 0.79 NG; percentage in repair ranged from 2.2 to 7.2% (Fig. 2A). In cultures treated with 0.01 mm BP (Fig. 2B), average NG ranged from 13 to 84 with 67 to 88% in repair. Of the agents tested, the strongest response was observed with UV radiation (Fig. 2C); average NG ranged from 82 to 237 with 100% of the cells in repair.

DNA repair responses in secondary cultures of contact-inhibited and growth factor-deprived HMEC are presented in Tables 1 and 2, respectively. Medium and DMSO controls were combined since statistical analyses showed no difference between the two control groups in NG or in repair. In general, for the one case examined, UDS did not differ between contact-inhibited or growth factor-deprived cultures. Because of limited material, extensive comparisons could not be made.

HMEC from all women examined had metabolic activation and DNA repair capabilities (Table 2). The degrees of the UDS responses were dose dependent. Qualitatively, the DNA repair responses did not differ between women, with the exception of 0.001 mm BP where no response was observed in two of the five women (growth factor-deprived Cases B402 and B499). Contact-inhibited Case B402 did, however, respond to 0.001 mm BP.

Indirect-acting genotoxictants activated via different metabolic pathways (BP, AFB1, 1,6-DNP, and 2-AAF) produced a positive response in secondary cultures of HMEC (Tables 1 and 2). BP, DMBA, AFB1, and TSC were negative or only slightly positive in passage 9 cultures of HMEC (Table 3). A direct-acting agent, UV radiation, produced a positive response in HMEC from all women examined, including passage 9. Of the two PAH tested in secondary cultures, BP induced a strong

Fig. 1. Histograms of percentage of HMEC with the indicated numbers of net nuclear grains. Net nuclear grains were quantitated as described in "Materials and Methods." Control cultures of HMEC (•); a total of 616 media and DMSO control cells were counted from 5 different women. HMEC exposed to 2.5% TSC (□); a total of 61 cells were counted from Case B499 exposed to 2.5% TSC. HMEC exposed to 0.01 mm AFB1 (○); a total of 61 cells were counted from Case 3014 exposed to 0.01 mm AFB1.
positive response in all cases whereas DMBA produced only a slight response in two cases (B402 and 3085; Table 2). No UDS activity was seen with TCDD (Table 1).

DISCUSSION

In view of the increasing incidence of human breast cancer and the possible role of environmental factors, it is important to identify environmental agents that are genotoxic in HMEC. Since carcinogens often exhibit species- and tissue-specific differences in factors such as biotransformation, the most relevant mechanistic information that is useful for risk assessment may be derived from test systems that utilize the actual human tissue at risk. Toward this end, we have developed an assay for measuring DNA repair in secondary cultures of HMEC. This assay provides a qualitative indication of potential genotoxic activity in these cells.

The methods for establishing and cultivating HMEC have been documented (20–22). These cultures are characterized with respect to epithelial- and mammary-specific properties (20, 23) and metabolism and DNA binding of PAH (24, 25). An important aspect of the HMEC UDS assay was the ability to control proliferation of the HMEC by either contact inhibition or growth factor deprivation. For the most part, no differences in DNA repair responses were observed for the two methods for reducing S phase. The low concentration of BP and AFB produced a positive response in contact-inhibited, but not in growth factor-deprived cultures of Case B402. Other cases, however, did respond to the same dose of the same agents when deprived of growth factors. Thus, this difference does not appear to be a function of these culture conditions.

No differences in the DNA repair response were observed among the samples from different women, except for growth factor-deprived cultures exposed to the low concentration of BP. Here, Cases B402 and B499 did not respond to 0.001 mM BP, whereas the other three cases responded in a positive manner. B402, however, did respond when contact inhibited. Thus, this difference does not appear to be due to interindividual variation.

In the present study, a wide variety of agents were examined to determine their ability to induce UDS in HMEC. The results reflect the metabolic activation and excision-repair capabilities of the HMEC. Positive responses were observed with chemicals requiring metabolic activation: BP, AFB, 1,6-DNP and 2-AAF. BP is a ubiquitous environmental agent of a chemical class known to induce mammary tumors in rats (5, 6). AFB is

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>NG ± SE ( ^a )</th>
<th>% in repair ± SE ( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP</td>
<td>0.001 mM</td>
<td>24.0 ± 4.2 ( ^c )</td>
<td>76 ± 6 ( ^d )</td>
</tr>
<tr>
<td></td>
<td>0.01 mM</td>
<td>25.0 ± 6.5 ( ^c )</td>
<td>84 ± 6 ( ^d )</td>
</tr>
<tr>
<td>AFB ( ^{1} )</td>
<td>0.001 mM</td>
<td>33.0 ± 37.0 ( ^e )</td>
<td>72 ( ^d )</td>
</tr>
<tr>
<td></td>
<td>0.01 mM</td>
<td>63.0 ± 57.0 ( ^e )</td>
<td>90 ( ^d )</td>
</tr>
<tr>
<td>TSC</td>
<td>0.25%</td>
<td>-6.8 ± 23.0 ( ^f )</td>
<td>20 ( ^d )</td>
</tr>
<tr>
<td></td>
<td>2.5%</td>
<td>8.2 ± 15.0 ( ^f )</td>
<td>33 ± 11 ( ^d )</td>
</tr>
<tr>
<td>UV</td>
<td>1000 J/m(^2)</td>
<td>237.0 ± 83.0 ( ^g )</td>
<td>100 ± 0 ( ^d )</td>
</tr>
<tr>
<td>Ethylmethanesulfonate</td>
<td>10.0 mM</td>
<td>55.0 ± 14.0 ( ^h )</td>
<td>100 ± 0 ( ^d )</td>
</tr>
<tr>
<td>1,6-DNP</td>
<td>0.0005 mM</td>
<td>7.1 ± 30.0 ( ^i )</td>
<td>40 ( ^d )</td>
</tr>
<tr>
<td></td>
<td>0.005 mM</td>
<td>30.0 ± 22.0 ( ^i )</td>
<td>84 ( ^d )</td>
</tr>
<tr>
<td>2-AAF</td>
<td>0.1 mM</td>
<td>29.0 ± 53.0 ( ^j )</td>
<td>60 ( ^d )</td>
</tr>
<tr>
<td>TCDD</td>
<td>0.000001 mM</td>
<td>-47.0 ± 37.0 ( ^k )</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.00001 mM</td>
<td>-33.0 ± 32.0 ( ^k )</td>
<td>8</td>
</tr>
</tbody>
</table>

\( ^{a} \) NG for at least 25 cells for each of 2 slides in 2 experiments; SE is slide-to-slide variation.

\( ^{b} \) An individual cell with >6 NG was considered in repair.

\( ^{c} \) Significantly greater than control at \( P < 0.01 \); Student's \( t \) test.

\( ^{d} \) Significantly greater than control at \( P < 0.05 \); \( ^{e} \) \( ^{d} \) test.

\( ^{e} \) NG for at least 25 cells for only 1 slide; variation is cell-to-cell.

\( ^{f} \) Judged as positive using the criterion of a population average NG value of >6 NG.
a naturally occurring fungal metabolite that has been detected in human breast milk (26). 1,6-DNP is a mutagenic environmental pollutant found in diesel exhaust, effluents from coal-fired power plants, and cigarette smoke (27, 28). It is a polycyclic aromatic nitro compound and potent inducer of DNA repair in rat and human hepatocytes (29). 2-AAF, a mouse liver carcinogen, induced a positive response in the rat and human hepatocytes (29). 2-AAF, a mouse liver carcinogen, induced a positive response in the rat and human hepatocytes (29). 2-AAF, a mouse liver carcinogen, induced a positive response in the rat and human hepatocytes (29). 2-AAF, a mouse liver carcinogen, induced a positive response in the rat and human hepatocytes (29).

Later passage HMEC (Case 184, Passage 9) did not respond to chemicals requiring metabolic activation. The enzymatic capability of mammalian cells often diminish substantially soon after they are placed in culture and with increased passage number (30). Secondary cultures of HMEC, however, retain the same metabolic capabilities as primary cultures (24). UV radiation was positive in Passage 9 cultures, suggesting that later passage HMEC may lose their metabolic activity while retaining DNA repair capabilities.

TSC produced a weak but positive response in secondary cultures of HMEC. TSC contains numerous components and is genotoxic in a variety of test systems (31). Nicotine and its metabolite continine are detected in breast milk of mothers who smoke (32, 33). Epidemiology data to date do not support a causal relationship between smoking and breast cancer (34). However, these studies have considered only smokers and non-smokers. When age at start of smoking was examined, a 30% increase in relative risk was found for women who started smoking prior to age 17 years (35). These results are consistent with radiation-induced breast cancer, in which the greatest risk is among women under 20 years of age at the time of exposure (36). Furthermore, exposure to passive smoke is suggested to be a major risk factor for breast cancer (4). Taken together, these data suggest an additional potential health risk to women from cigarette smoke, particularly when exposure to tobacco smoke occurs during childhood.

Comparisons of the DNA repair response in HMEC to two PAH revealed BP to be a much stronger inducer of UDS than an equimolar concentration of DMBA. These data correlate with in vitro mutagenicity and DNA binding levels in HMEC (23–25). When in vitro mutagenicity and DNA binding levels of BP and DMBA were compared in rat mammary epithelial cells, a pattern opposite to that of HMEC was obtained which correlates with the carcinogenic activity of DMBA in the rat mammary gland (23–25). DMBA is a more effective mammary carcinogen than BP in rats (37). When sister chromatid exchange induced by BP and DMBA was compared in human and rat mammary epithelial cells, results confirmed the notion that BP has a greater genotoxic effect than DMBA in HMEC (38). The existence of DNA adducts in HMEC in situ has been demonstrated; human and rat mammary epithelial cells form different DNA adducts when exposed to BP in vitro (39). The differential UDS response observed with BP and DMBA in HMEC, together with the previous studies mentioned above, suggest that caution should be applied when extrapolating rodent data to humans in the absence of mechanistic information and that the potential involvement of BP in human breast cancer should be considered.

TCDD has been detected in human breast milk and fat (40, 41). This compound is a very potent carcinogen in rodents and appears to exert its carcinogenic effect primarily through non-genotoxic mechanisms (42). Recent epidemiological studies are not inconsistent with the hypothesis that TCDD is a human carcinogen (43, 44). TCDD did not produce a DNA repair.

Table 2 DNA repair response in growth factor-deprived secondary cultures of HMEC

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>NG ± SE</th>
<th>% in repair ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP</td>
<td>0.001 mm</td>
<td>0.7 ± 0.1</td>
<td>8 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.01 mm</td>
<td>1.8 ± 0.1</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>DMBA</td>
<td>0.001 mm</td>
<td>0.2 ± 0.1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.01 mm</td>
<td>0.3 ± 0.1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>AFB1</td>
<td>0.001 mm</td>
<td>0.3 ± 0.1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.01 mm</td>
<td>0.4 ± 0.1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>TSC</td>
<td>0.5%</td>
<td>0.6 ± 0.1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>2.5%</td>
<td>0.8 ± 0.1</td>
<td>7 ± 1</td>
</tr>
</tbody>
</table>

Table 3 DNA repair response in growth factor-deprived passage 9 HMEC

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>NG ± SE</th>
<th>% in repair ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP</td>
<td>0.001 mm</td>
<td>0.7 ± 0.1</td>
<td>8 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.01 mm</td>
<td>1.8 ± 0.1</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>DMBA</td>
<td>0.001 mm</td>
<td>0.2 ± 0.1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.01 mm</td>
<td>0.3 ± 0.1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>AFB1</td>
<td>0.001 mm</td>
<td>0.3 ± 0.1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.01 mm</td>
<td>0.4 ± 0.1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>TSC</td>
<td>0.5%</td>
<td>0.6 ± 0.1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>2.5%</td>
<td>0.8 ± 0.1</td>
<td>7 ± 1</td>
</tr>
</tbody>
</table>
response in HMEC. However, cytoplasmic grains were increased resulting in a much lower NG count. The biological basis for the TCDD-induced increase in cytoplasmic grain counts in HMEC is unknown.

In conclusion, this study demonstrates the use of HMEC for assessing chemically induced DNA repair as measured by UDS. The HMEC UDS assay will be useful in addressing the role of environmental agents in human breast cancer and aiding in the validation of proposed animal models.

ACKNOWLEDGMENTS

We thank Dr. Martha Stamper for the 184 cell strain and Dr. Thomas Sutler for helpful discussions.

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Genotoxicity of Environmental Agents in Human Mammary Epithelial Cells

Sandra R. Eldridge, Michael N. Gould and Byron E. Butterworth


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