Predisposition of Cloned Fetal Hamster Lung Epithelial Cells to Transformation by a Precarcinogen, Benzo(a)pyrene, Using Growth Hormone Supplementation and Collagen Gel Substratum

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ABSTRACT

A cloned fetal Syrian hamster lung epithelial cell line (M3E3/C3) was used to compare the influence of two different culture conditions on the degree of cellular differentiation and susceptibility of the cells to undergo malignant transformation by a precarcinogen, benzo(a)pyrene. Conventional conditions consisted of growth medium containing Roswell Park Memorial Institute Medium 1640, pyruvate, and fetal bovine serum and a substratum of plastic. Complex conditions comprised the growth medium supplemented with insulin, hydrocortisone, estradiol, epidermal growth factor, transferrin, and cholera toxin and a substratum of collagen gel. Under the complex culture conditions, there was extensive development of endoplasmic reticulum and Golgi vesicles, whereas under conventional conditions these organelles were only minimally developed. This was correlated with 1.5-1.8 times enhancement of ethoxyccoumarin deethylase and reduced nicotinamide adenine dinucleotide phosphate-dependent cytochrome c reductase activities. Deposition of added benzo(a)anthracene into water-soluble compounds increased with the period of incubation and reached about 40% of initial benzo(a)anthracene (50 µg/10 ml/flask) at 48 h under the complex conditions, whereas under conventional conditions only <4% decomposition occurred.

Benzo(a)pyrene in the dose range 2-8 µg/ml was strongly cytotoxic and caused significant anchorage independent transformation only under complex culture conditions. Transformed cells produced tumors in two of four hamsters during 8 months following s.c. injection within 48 h of birth. These results suggest that the complex culture conditions predisposed the cloned fetal epithelial cells to malignant transformation by benzo(a)pyrene through stimulation of cellular differentiation and development of enzyme systems capable of activating it metabolically.

INTRODUCTION

In previous papers (1, 2) we reported that cloned Syrian hamster fetal lung epithelial cells (line M3E3/C3) were transformable under simple conventional culture conditions by a direct-acting carcinogen, MNNG, but not by an indirect-acting precarcinogen, BaP. The probable reason for this difference is that cells of the established line do not possess enzymes capable of metabolizing the precarcinogen BaP to the active carcinogen. This is consistent with their derivation from fetal respiratory epithelium and the number of serial passages necessary to establish a cell line by single cell cloning procedures.

Studies with hepatocytes have shown that they rapidly lose the capability for xenobiotic metabolism under conventional culture conditions (3, 4) but that there is much better maintenance of this activity when cells are grown on collagen membranes (5) or in hormone-supplemented medium (6-8). Following this lead, we have recently found that the hamster fetal epithelial cell line responds to growth on collagen gel in a medium supplemented by hormones and vitamin A by developing abundant ER and evidence of secretory activity. Since the ER is known to be a subcellular compartment heavily involved in xenobiotic metabolism by cytochrome P-450-related enzymes, we decided to evaluate the predisposing effect of complex culture conditions on the in vitro toxicity and transformation due to BaP in the cloned fetal hamster lung epithelial cells. As far as we are aware, this is the first report to demonstrate that a cloned fetal respiratory cell line, which is otherwise unresponsive to a precarcinogen, BaP, can be rendered transformable by BaP through cultivation in a medium supplemented with various hormones and on a collagen gel substratum.

MATERIALS AND METHODS

Reagents and Culture Vessels. Sodium pyruvate, CaCl2, vitamin B12, trace elements, l-asparagine, and histochemical electron microscopical stains were purchased from Merck, Darmstadt, Federal Republic of Germany. Hydrocortisone sodium salt, 17β-estradiol, human transferrin, cholera toxin, DEAE-dextran, hypoxanthine, 2-mercaptoethanol, and reagents for NADPH-dependent cytochrome c reductase measurements were from Sigma, Munich, Federal Republic of Germany. Penicillin, streptomycin and 8-cm² plastic dishes were from Gibco Europe-Nunc, Wiesbaden, Federal Republic of Germany, RPMI 1640, Vitrogen 100, and fetal bovine serum (tested for Mycoplasma) were from Flow Laboratories, Meckenheim, Federal Republic of Germany. EGF, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, DMSO, collagenase (purest), and Taka diastase were from Boehringer, Mannheim, Federal Republic of Germany. Insulin and 7-hydroxycoumarin were from Fluka, Neu-Ulm, Federal Republic of Germany. Bacto-agar and tryptose phosphate broth were from Difco, Detroit, MI. [14C]dThd (55-56 mCi/mmol) was from Amersham Buchler, Braunschweig, Federal Republic of Germany. Plastic dishes (20 cm²) and T-flasks (25 and 75 cm²) were from Becton-Dickinson, Heidelberg, Federal Republic of Germany. BaP and BaA were provided by Professor G. Grimmer, Biochemisches Institut für Umweltcarcinogene, Abhensburg, Federal Republic of Germany. MNNG was supplied by Professor R. Preussmann, Deutsches Krebsforschungszentrum, Heidelberg, Federal Republic of Germany.

Nutrient Media and Cell Line. The growth medium consisted of 20% fetal bovine serum and 80% RPMI 1640 supplemented with glucose (3 mg/ml), sodium pyruvate (110 µg/ml), FeSO4·7H2O (0.46 µg/ml), vitamin B12 (1.2 µg/ml), penicillin (100 units/ml), and streptomycin (100 µg/ml). The hormone-supplemented medium contained the growth medium (98.1 ml), insulin (0.8 mg), hydrocortisone sodium salt (0.18 mg), 17β-estradiol (10⁻⁹ mol), EGF (2 µg), human transferrin (2.5 mg), cholera toxin (0.1 mg), CaCl2 (5.16 mg), and trace elements according to McKeohan et al. (9). The establishment of the fetal hamster lung epithelial cell line M3E3/C3 has been previously described (1). Frozen stock cells were quickly thawed and maintained in a 75-cm² T-flask containing the growth medium at 37°C with a saturated humidity and 10% CO₂. Throughout the experiments cells were used at the passages from 30-35. Regular checking of the cell line for Mycoplasma contamination always proved negative.

1 M. Emura, U. Mohr, M. Riebe, M. Aufderheide, and D. Dungworth. Regulation of growth and differentiation by vitamin A in a cloned fetal lung epithelial cell line cultured on collagen gel and in hormone-supplemented medium, manuscript in preparation.

Received 6/2/86; revised 10/10/86; accepted 11/7/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This study was partly supported by a grant for Humanisierung des Arbeitslebens.

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4 The abbreviations used are: MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; AI, anchorage independent; BaA, benzo(a)anthracene; BaP, benzo(a)pyrene; DMSO, dimethyl sulfoxide; ECD, ethoxyccoumarin deethylase; EGF, epidermal growth factor; ER, endoplasmic reticulum; RGE, relative growth efficiency; dThd, thymidine.
Cell Culture and Transformation with BaP. Under the conventional conditions, cells were plated directly on the plastic surface of culture vessels and fed with the growth medium. By contrast, under the complex conditions cells were plated on collagen (Vitrogen) gel (10) with 0.003% gelatin and cultured in the growth medium for the first 4 days; then the medium was switched to the hormone supplemented one (Fig. 1). The temperature, humidity, and CO₂ concentration were as described for the maintenance culture in the previous section.

For the transformation experiments with BaP or MNNG under conventional conditions, 5 x 10⁵ cells were plated in 20-cm² dishes. One day later, BaP dissolved in DMSO (final concentration, 0.3%) was added at the prescribed doses and kept in cultures for 3 days. The other procedures were the same as for the experiments under the complex conditions as illustrated in Fig. 1 except for the trypsinization (trypsin, 0.25% plus EDTA, 0.02%) on day 14 instead of using 0.8% collagenase and the use of soft agar without EGF.

For the transformation experiments under the complex conditions, BaP treatment was carried out for 4 days from days 6-10 (Fig. 1) in cultures consisting of 5 x 10⁴ cells initially plated on the collagen gels prepared in 8-cm² dishes. On day 14 cells were detached from the collagen gel with 0.8% collagenase (45 min incubation at 37°C) and transferred into 25-cm² flasks with regular growth medium. After trypsinization on day 18, fractions of cells (1 x 10⁵ cells/20-cm² dish) from each flask were plated in soft agar for AI colony detection, and the other fractions were plated again in 25-cm² flasks for the second AI test on day 24 (see Fig. 1) except that during BaP treatment the appropriate culture medium was changed every 2 days. Soft agar culture was carried out using a medium containing L-asparagine (1.8 nig/in I). The appropriate culture medium was changed every 2 days. Soft agar culture was carried out using a medium containing L-asparagine (1.8 nig/in I).

RESULTS

The susceptibility of the cells of the M3E3/C3 line to the toxicity and transforming effects of BaP was reexamined in the first place under the conventional conditions. As expected from previous studies (2), BaP treatment induced no significant AI transformation in the dose range up to 10 µg/ml (Table 1). There was no clear dose-dependent reduction in relative colony-forming efficiency (Table 1) but slight reduction in size of colonies with increasing dose was observed. In contrast, the direct carcinogen MNNG caused a clearly dose-dependent reduction of colony-forming efficiency (Table 1) with significant colony size reduction at doses between 0.05 and 1.0 µg/ml. It also caused transformation at 1.0 µg/ml (30-50 AI colonies/10⁵ plated cells), although at lower doses no transformation
was detected. Then the toxicity and transforming activity of BaP was examined under the complex conditions (Table 2). The 2 \( \mu \)g/ml dose reduced the growth to 14% of the DMSO control (compare RGE). Although the parameters for measuring reduction in cell growth were different in Tables 1 and 2 (relative colony-forming efficiency compared to RGE), the general indication was that the toxic effect of BaP was greater under complex culture conditions. In addition, in the complex culture, statistically significantly more AI colonies were produced than in the control \( (P < 0.05) \). Increase in BaP dose to 4–8 \( \mu \)g/ml considerably increased the number of AI colonies, although the difference was statistically insignificant because of the large variations. It is well known in mammalian cell mutagenesis that there is an optimal culture period after mutagen treatment for the most effective expression of mutated phenotypes (17). This phenomenon was demonstrated in our system. Prolongation of the post-treatment period by 6 days resulted in good restoration of RGE (>90% of RGE). Although the total number of AI colonies observed after the 14-day posttreatment period was greater (>3 times at 2 and 4 \( \mu \)g/ml BaP) than after 8 days, the transformation frequency corrected for RGE (data not shown) was higher after 8 days.

To better understand the possible mechanisms involved in the predisposition to transformation by BaP, stages of cellular differentiation and levels of relevant marker enzyme activities were compared between the conventional and complex conditions after 5–10 days of culture. Under complex culture conditions, differentiation of ER become increasingly prominent from days 5–6 onwards and there was a parallel increase in profiles of Golgi apparatuses (Fig. 2). The very much elongated and broadened rough ER cisternae usually contained short fibrillar materials, and small mucus-like granules were occasionally seen. By contrast, in the conventional conditions, no such differentiation of ER and Golgi apparatuses was detected and no mucus-like granules occurred. When cells grown under complex conditions are transferred to conventional growth medium, they return to the morphological appearance of cells maintained in conventional conditions. In good correlation with the morphological findings, the basal (not induced) levels of both NADPH-dependent cytochrome c reductase and ECD were considerably higher under complex culture conditions than under conventional conditions. Activities were increased from 2.5–3.8 nmol/min/mg whole cell protein (range of variation, <5%; cytochrome c reductase) and 11.9 ± 0.2 (SD)–21.7 ± 7.0 pmol/20 min/mg whole cell protein (ECD) when measured on day 9 in conventional as compared to complex culture conditions.

In order to assay for carcinogen metabolism, BaA was incubated with cells for different periods under conventional and complex conditions and the amount of unmetabolized BaA recovered in the water-insoluble phase was compared (Table 3). BaA is very much less cytotoxic than BaP, so BaA was chosen to avoid the influence of greatly reduced cell numbers on the metabolic capacity per flask. In addition we have the technical guarantee for the almost 100% recovery of initial BaA. The results clearly showed that growth in supplemented medium on collagen gel yielded the most efficient decomposition of BaA. It is also noteworthy that collagen gel alone stimulated some degree of BaA decomposition. Although it is unknown at present which components of the complex conditions are most critical, it is very important to analyze these components in view of better characterization of the cellular response.

Another aspect of cellular activity, i.e., proliferation, can also influence the susceptibility of cells to chemical transformation. Therefore, the increase in cell number and the rate of \([^{14}C]dThd \) uptake in the acid-precipitable fraction were examined at 2-day intervals up to 12 days under the complex culture conditions (Table 4; see also Fig. 1). Under complex conditions, the cells did not grow exponentially as they usually do under conventional conditions, but they grew almost in a linear fashion. The

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**Table 1** Growth ability of M3E3/C3 cells following treatment with BaP or MNNG in conventional culture conditions as expressed in relative colony-forming efficiency

<table>
<thead>
<tr>
<th>BaP</th>
<th>Transformation ((\times 10^{-4}))</th>
<th>No. of colonies/dish</th>
<th>RCFE</th>
<th>( P^* )</th>
<th>MNNG</th>
<th>Transformation</th>
<th>No. of colonies/dish</th>
<th>RCFE</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (DMSO)</td>
<td>3</td>
<td>355.1 ± 27.1</td>
<td>100</td>
<td>0.01</td>
<td>0.05</td>
<td>9 \times 10^{-4}</td>
<td>337.6 ± 29.2</td>
<td>92</td>
<td>Not significant</td>
</tr>
<tr>
<td>1.0</td>
<td>3</td>
<td>299.6 ± 16.7</td>
<td>84</td>
<td>0.01</td>
<td>0.05</td>
<td>9 \times 10^{-4}</td>
<td>251.7 ± 26.5</td>
<td>71</td>
<td>0.002</td>
</tr>
<tr>
<td>5.0</td>
<td>5</td>
<td>279.6 ± 17.6</td>
<td>79</td>
<td>Not significant</td>
<td>0.1</td>
<td>9 \times 10^{-4}</td>
<td>21.7 ± 17.5</td>
<td>10</td>
<td>0.0005</td>
</tr>
<tr>
<td>10.0</td>
<td>5</td>
<td>292.7 ± 17.5</td>
<td>82</td>
<td>Not significant</td>
<td>1.0</td>
<td>3–5 \times 10^{-3}</td>
<td>114.1 ± 44.9</td>
<td>32</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

* Frequency of AI colonies in percentage by taking into account the number of cells \((10^4–10^5)\) plated per soft agar dish.

* RCFE, relative colony-forming efficiency, calculated by taking the control colony-forming efficiency (colony number \( \times 100 \)/plated cell number) as 100.

* Student's \( t \) test carried out between neighboring doses.

* Mean ± SD of 5 dishes.

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**Table 2** Dose relationship of growth ability and anchorage independency in M3E3/C3 cells 8 days after BaP treatment in complex culture conditions

<table>
<thead>
<tr>
<th>BaP (( \mu )g/ml)</th>
<th>No. of cells/</th>
<th>Growth efficiency</th>
<th>RGE</th>
<th>Total AI colonies/dish</th>
<th>Relative AI colony no.</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dish ((\times 10^4))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (DMSO)</td>
<td>2.28 ± 0.52</td>
<td>45.6</td>
<td>100</td>
<td>8.7 ± 0.3</td>
<td>9</td>
<td>0.0005</td>
</tr>
<tr>
<td>2</td>
<td>0.33 ± 0.04</td>
<td>6.6</td>
<td>64</td>
<td>28.3 ± 5.9</td>
<td>202</td>
<td>Not significant</td>
</tr>
<tr>
<td>4</td>
<td>0.24 ± 0.04</td>
<td>11.1</td>
<td>95.7 ± 43.5</td>
<td>870</td>
<td>Not significant</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.23 ± 0.05</td>
<td>4.6</td>
<td>10</td>
<td>77.3 ± 53.5</td>
<td>773</td>
<td>Not significant</td>
</tr>
</tbody>
</table>

* Number of cells recovered at the time of the first soft agar plating. Cells were plated on collagen gel in the amount of \( 5 \times 10^4 \)/dish at the start of the experiment. Thereafter no cells were discarded.

* Ratio of the number of cells recovered to the plated cell number \((5 \times 10^4)/\)dish on the collagen gel.

* Growth efficiency for the DMSO control was taken as 100. This index indicates the growth ability of the cell population treated with various BaP doses.

* Number of AI colonies larger than 0.12 mm diameter grown in 27 days of soft agar culture. Each dish was derived from the individual original triplicate.

* Corrected with regard to RGE, assuming that BaP-treated cells have the same growth efficiency as that of the control. The formula was total colonies/dish \( \times 100 \)/RGE.

* Student's \( t \) test carried out between neighboring doses.

* Average ± SE of triplicate dishes.
population doubling time was calculated as 55 h at the initial phase of growth on gels, then becoming much longer with the time of cultivation (Table 4), while that of logarithmic growth on the plastic was constantly 11 h (see Ref. 1). dThd uptake generally decreased with the time of incubation but a steep temporary rise to as high as the level of day 2 was observed on day 8, which corresponded to the midterm of the BaP treatment in the case of transformation experiments. Finally, to link AI growth with malignant transformation, several AI colonies from each dose group were propagated as monolayer cultures. Two of 4 animals given injections from a BaP-treated colony and none of 6 animals given injections from a DMSO-treated colony developed tumors in the injection sites within 32 weeks after injection.

Table 3 Recovery of unmetabolized BaA after various periods of incubation under different culture conditions

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Unmetabolized BaA (µg/flask)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6-h incubation</td>
</tr>
<tr>
<td>Conventional medium</td>
<td>49.3*</td>
</tr>
<tr>
<td>Conventional on collagen gel</td>
<td>42.9</td>
</tr>
<tr>
<td>Supplemented medium on collagen gel (complex)</td>
<td>48.8</td>
</tr>
</tbody>
</table>

* Mean of duplicate determinations.

DISCUSSION

This work has shown that the cells of a permanent epithelial cell line, which are unresponsive to transformation by BaP under conventional culture conditions, can be predisposed to transformation by BaP through manipulation of culture conditions. Transformation was manifest by AI growth and preliminary studies have indicated the capability of AI colonies to produce tumors after transplantation in newborn hamsters. The tumors were diagnosed light microscopically as poorly differentiated carcinomas similar to those induced by MNNG (1). Electron microscopic studies are under way.

Although a variety of mechanisms might be responsible for acquisition of transformability under complex culture conditions, there is strong evidence to indicate that it is causally related to the development of enzymes capable of metabolizing the precarcinogen BaP to its active carcinogenic metabolite. The most direct evidence is that complex culture conditions were associated with enhanced basal levels of ECD and NADPH cytochrome c reductase, which are markers of increased capability for BaP metabolism. This was correlated with increased capacity of the cells to metabolize BaA, a closely related polycyclic hydrocarbon. Very strong conjugating activities developed in these cells which prevented easy detection of single BaA metabolites in the spent medium. The morphological correlates were the development of ER, Golgi apparatus, and occasional mucus-like secretory granules. In the respiratory

Table 4 Rate of cell growth and thymidine uptake of M3E3/C3 cells under complex culture condition

<table>
<thead>
<tr>
<th>Days of culture</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell no./dish (x 10³)</td>
<td>5</td>
<td>10.3 ± 2.6*</td>
<td>13.7 ± 2.6</td>
<td>19.3 ± 5.8</td>
<td>22.3 ± 5.2</td>
<td>26.0 ± 5.2</td>
<td>32.3 ± 5.7</td>
</tr>
<tr>
<td>[³H]dThd (x 10⁴ dpm/cell)</td>
<td>Not done</td>
<td>27.3 ± 9.9</td>
<td>20.3 ± 2.3</td>
<td>15.8 ± 7.6</td>
<td>26.0 ± 11.9</td>
<td>10.0 ± 4.0</td>
<td>8.5 ± 2.4</td>
</tr>
</tbody>
</table>

* Mean ± SE of the average counts of duplicate cultures run in 3 separate complete experiments.
Epithelium, except in Clara cells, rough ER dominates over smooth ER in amount. However, there is good evidence that the function of cytochrome P-450 is very similar in both the smooth and rough ER, although the activity is stronger in the former than in the latter (18). Various components of our complex culture system such as hormones, EGF, and collagen gel have also been found by others to enhance the level of cytochrome P-450/monooxygenase system enzymes in various cell cultures (5, 6, 8, 19–27).

It should be noted that predisposition to transformation was associated with evidence of functional differentiation, not with proliferative activity in general, because under complex culture conditions the cells had a linear growth response in contrast to the logarithmic growth observed under conventional conditions. Population doubling was also much slower in the complex conditions than in the conventional conditions. In addition, the dThd uptake decreased on the whole in the complex conditions. However, on day 8 of our culture regimen we observed a small but distinct peak of dThd uptake into the cells. It is unclear whether this represented normal DNA replication or, at least partly, another type of DNA synthesis such as amplification. The former seems less likely than the latter, since there was no sign of particularly stimulated cell growth after this period. Gene amplification is usually common in functionally differentiating cells. It is conceivable that such active replication of any damaged DNA sequences, although transient, is also apt to give rise to miscoding of genetic information as does regular DNA replication (28). In our cells cultured under complex conditions, BaP exposure was carried out in the period including this rapid transient DNA replication and therefore similar mechanisms would have had also contributed to their acquisition of predisposed nature. In this respect it should be interesting to study any enhancing effects of complex conditions on the transformation by MNNG.

The combination of components in the complex culture conditions caused the undifferentiated epithelial cell line to develop abundant rough ER, well-developed Golgi vesicles, and occasional small secretory granules. These morphological characteristics therefore give them a resemblance to small mucous granule cells which have been postulated to be the most likely carcinogen target cell candidate in animal and human respiratory epithelium (29, 30). Further studies are under way to determine whether the partly differentiated cell in vitro is a useful analogue of the carcinogen target cell in vivo.

A final aspect to be considered is the relatively high background of AI colonies in DMSO controls grown in soft agar with EGF. The EGF in the soft agar medium seems to play a stimulatory role as reported by others (31). Our preliminary experiments have shown more than 10-fold differences in the frequency of AI colonies between EGF-supplemented and -unsupplemented soft agar cultures.

The most important conclusion to be drawn from this work is that just as the bacterial mutagenesis system requires addition of enzymes needed to activate indirect-acting mutagens (carcinogens), so an in vitro cell transformation assay requires cells that are enzymatically capable of activating precarcinogens to be tested in the system.

ACKNOWLEDGMENTS

We are very grateful to Professors G. Grimmer and J. Jacob, Biochemisches Institut für Umweltcarcinogene, Ahrensburg, Federal Republic of Germany, for the gas chromatography analysis. We also appreciate the technical contribution of C. Blankenburg, K. Hoffmann, and S. Rosinke and assistance with the English language from S. Waring.

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