Altered Growth Properties of Chinese Hamster Cells Exposed to 1-Methylguanine and 7-Methylguanine

R. W. Trewyn and Sylvia J. Kerr

Department of Biochemistry, University of Colorado Medical Center, Denver, Colorado 80262

ABSTRACT

Primary Chinese hamster embryo cell cultures generally yield cell lines with a finite lifetime in culture. However, if early-passage cells are exposed chronically to either of two normal degradation products of transfer RNA, 1-methylguanine or 7-methylguanine, they are converted to continuous lines with altered growth characteristics and morphology. The continuous cell lines have saturation densities 2- to 10-fold higher than did finite control cultures, and some have the ability to grow in soft agar. Certain cultures have the general appearance of fibroblasts while others are more epithelial-like. Quantitative and qualitative alterations in the transfer RNA methyltransferases are early markers for neoplastic transformation in vivo and in vitro. Transfer RNA methyltransferase activity in the continuous lines is elevated compared to that of finite Chinese hamster cells. Neoplastic transformation has been demonstrated for a 1-methylguanine-derived line, and both 1-methylguanine- and 7-methylguanine-treated cell lines exhibit characteristics similar to those of Chinese hamster cells transformed with the carcinogen 3,4-benzopyrene or the DNA tumor virus SV40.

INTRODUCTION

The presence of a number of modified purines and pyrimidines as minor components of RNA has been observed as early as 1958. Since then the number and complexity of such modified components that have been identified has burgeoned (1).

The origin of synthesis of the minor bases in RNA was obscure until it was discovered that they are synthesized at the macromolecular level by specific enzymes. The first such enzymes to be characterized were the tRNA methyltransferases (10). These enzymes modify the structure of performed tRNA by the addition of methyl groups, with the high-energy compound S-adenosylmethionine as the methyl donor. They are base and sequence specific, as well as species and even organ and organelle specific (10). Thus they confer structural and conformational individuality on tRNA molecules.

The tRNA methyltransferases have been shown to undergo alterations, both quantitative and qualitative, in a number of biological systems that are being subjected to shifts in regulatory mechanisms (10) as well as in every tumor examined (1, 11). In addition elevated excretion of breakdown products of tRNA, including methylated bases and nucleosides, has been observed in cancer patients (1, 20) and in animal models. The increase in the excretion levels can be more than 10-fold, which is extraordinary in view of the small mass of the cancer relative to body mass. It is possible that the concentration of these components proximal to the cancer could be continuously elevated. It seemed to be of interest to study any response of normal cells to prolonged exposure to modified bases resulting from the breakdown of tRNA. Such a study has not been reported heretofore.

While examining the effects of 1-methylguanine and 7-methylguanine on primary cultures of normal Chinese hamster cells, we observed that these agents cause heritable changes in the cultured cells. These heritable changes include morphological, proliferative, and biochemical properties, and in some cases they correspond to in vitro carcinogenesis; i.e., the cells become tumorigenic.

MATERIALS AND METHODS

Cells and Culture Conditions. Primary cultures of Chinese hamster embryo cells (ChH)4 were prepared as described by Lehman and Defendi (13). Confluent cultures were split at a ratio of 1:4 at 5- to 7-day intervals. These cells generally survive 10 to 12 passages under the conditions used. The cells were maintained in MEM containing 10% fetal calf serum.

Duplicate treated and control cultures were maintained in every experiment, and the culture medium was supplemented with the agents to be tested at the first or second passage. The 2 modified purines were used at a concentration of 10 μM as was guanine, and supplementation was continued uninterrupted even after continuous cell lines were obtained. The carcinogenic hydrocarbon 3,4-benzopyrene was dissolved in dimethyl sulfoxide and was tested at 1 μM. The final dimethyl sulfoxide concentration in the medium was 0.5% as described by Chen and Heidelberger (2) in their in vitro transformation systems. Cells were exposed to benzopyrene for 14 days, during which time the cells were subcultured once.

A cloned SV40-transformed ChH line (clone 31) designated ChH:SV40 (24) was obtained from Dr. J. M. Lehman, Department of Pathology, University of Colorado Medical Center, as were 2 of the primary ChH cultures.

Characterization of Continuous Cell Lines. Monolayer

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1 The abbreviations used are: ChH, Chinese hamster finite cell lines; MEM, Eagle's minimal essential medium; ChH:SV40, Chinese hamster SV40-transformed continuous line; ChH-1G, Chinese hamster 1-methylguanine-exposed continuous lines; ChH-7G, Chinese hamster 7-methylguanine-exposed continuous lines; ChH-5P, Chinese hamster continuous line occurring spontaneously; ChH-BP, Chinese hamster 3,4-benzopyrene-treated continuous line.
cultures on coverslips were stained with eosin-methylene blue (May-Grünwald) and Giemsa, and were photographed.

Growth curves were established after cells were plated at 2 \times 10^4 cells/25-sq cm culture flask in 5 ml MEM plus 10% fetal calf serum. The medium for ChH-1G and ChH-7G cells was supplemented with 10 \mu M 1-methylguanine and 7-methylguanine, respectively. The culture medium was not changed for the duration of the experiment. At 2- and 3-day intervals duplicate cultures were trypsinized, and cells were counted with a hemacytometer.

Saturation densities were determined after cultures were split 1:2 and allowed to grow for 4 days. Duplicate cultures were again counted.

Growth in soft agar was established by the methods of MacPherson (14), with 10^4 cells plated in 60-mm Petri dishes.

Karyology of continuous cell lines was established as described by Lehman and Defendi (13). Giemsa blood stain was used to stain the chromosomes.

tRNA methyltransferase specific activity was determined as previously described (9), with Escherichia coli B tRNA as the substrate. Enzyme extracts were prepared as described by Wilkinson and Kerr (22), but with centrifugation at 30,000 \times g for 20 min.

**Chemicals and Reagents.** MEM and fetal calf serum were purchased from Grand Island Biological Co., Grand Island, N. Y. E. coli B tRNA was obtained from General Biochemicals, Inc., Chagrin Falls, Ohio, and S-adenosyl-L-[methyl-\(^{14}\)C]methionine was from Amersham/Searle Corp., Arlington Heights, Ill. 7-Methylguanine was purchased from Vega-Fox Biochemicals, Tucson, Ariz.

1-Methylguanine was prepared from 1-methylguanosine (Sigma Chemical Co., St. Louis, Mo.) by hydrolysis in 1 N HCl at 100° for 1 hr followed by lyophilization. Purity of the methylated bases was established by high-pressure liquid chromatography on a Whatman type PVC-1025 ODS column eluted with 0.05 M (NH\(_4\))\(_2\)HPO\(_4\), pH 5.1, containing 5% methanol, and the purity of each was greater than 98%.

**RESULTS**

Five primary Chinese hamster embryo cell cultures were used. Duplicate control cultures were established at the first passage of the primary, and from these 10 untreated cultures 1 continuous cell line (ChH-Sp,) arose spontaneously. Duplicates from 3 of the primary cultures were exposed to 10 \mu M 1-methylguanine, and all 6 cultures gave rise to continuous cell lines. Six continuous lines were also obtained from 6 cultures exposed to 10 \mu M 7-methylguanine. Two cultures exposed to 10 \mu M guanine did not give rise to continuous lines. Only 1 continuous cell line was derived from 2 cultures exposed to 3,4-benzopyrene.

During the first 6 to 8 passages of ChH cells, no differences were apparent in treated and untreated cells. The cells in the medium containing 10 \mu M 1-methylguanine or 7-methylguanine grew at the same rate as did the controls. During later passages when the generation time of ChH cells lengthens, the treated cells began to grow more rapidly. The transition from finite to continuous growth was generally smooth and gradual, but growth of individual clones (15) was sometimes observed when the majority of the cells had stopped growing. After establishment of continuous lines, the cells maintained their morphological and growth characteristics if the test compounds were removed from the medium. This has been verified by leaving the methylated base out for up to 40 passages with some cultures, but as a general practice the agents were included continuously.

The growth orientation and cell morphology of 3 long-term continuous lines are shown in Fig. 1 at both high and low cell densities. These 3 cell lines were derived from the same primary culture, but they differ greatly in appearance. The ChH-Sp,, cells exhibit an oriented growth pattern and form a confluent monolayer, whereas ChH-1G2 cells are more disoriented and overgrow extensively. The ChH-7G3 cells form a confluent monolayer, but their morphology is distinct from that of the other 2 lines. The ChH-7G3 cells are epithelial-like and the ChH-Sp, cells and ChH-1G2 cells have the appearance of fibroblasts. All lines derived from 1-methylguanine exposure do not have a fibroblast-like morphology; similarly, lines arising from exposure to 7-methylguanine are not invariably epithelial-like, but the morphologies and growth orientations are generally similar to one of those shown in Fig. 1.

Growth curves for ChH-Sp,, ChH-1G2, and ChH-7G3 cells plated at low density are shown in Chart 1. The treated cell lines rapidly commence proliferation, whereas the spontaneous line requires a lengthy adaptation period. The ChH-Sp, cells also stop growing much sooner, indicating more stringent nutritional requirements. In no case did the cultures attain confluency under the conditions used.

Saturation densities for finite ChH cells and all continuous cell lines were determined; the results are shown in Table 1. All of the continuous lines had saturation densities higher than did the finite line, but the variation among the continuous lines was more than 4-fold.

Growth in soft agar was examined for all of the cultures, and these results are also presented in Table 1. Cell lines with the ability to grow in soft agar were ChH-1G2, ChH-1G, ChH-7G3, ChH-BP, and ChH:SV40. This ability was not endowed by the primary culture from which they were derived and did not necessarily correlate to their saturation densities.

A cytogenetic analysis of the continuous cell lines was...
Aberrant Cell Growth Induced by Methylated Guanines

undertaken, and the ChH-Sp line was the only one with a diploid (2n = 22) karyotype. ChH-1G cells were all found to be hyperdiploid with a modal chromosome number of 23, and the ChH-7G3 and ChH-7G6 lines were hypodiploid with a modal chromosome number of 21. A single chromosome number did not predominate in ChH-7G, ChH-7G6, or ChH-BP cells. An unstable, heterogeneous population has been observed for the ChH:SV40 line (24). Large chromosome fragments were observed in most treated lines, and these currently are being characterized.

Aberrant tRNA methyltransferase activity and capacity are useful biochemical markers for transformation (1, 11). The enzyme activity is also elevated in embryonic tissue (11) and preneoplastic tissues (21, 23). As primary Chinese hamster embryo cultures are passaged, the tRNA methyltransferase activity decreases in proportion to the number of passages, and in attenuated cell lines that will no longer divide it is extremely low (results to be published elsewhere). The enzymes were assayed in finite and continuous cell lines, and the results are presented in Table 2. The values derived from passage 4 cells of the finite cultures represent an intermediate value in the course of the decline of growth of such cultures. The specific activity of the enzymes was elevated in most of the continuous cell lines compared to ChH cells. There is a general correlation between activity and saturation density of the continuous lines and with those cell lines that grow to higher densities exhibiting higher activities. It has been shown previously, however, that tRNA methyltransferase activity is not necessarily related to growth rate (11).

Tumorigenicity of ChH-Sp, ChH-1G2, and ChH-7G3 cells was tested by injecting 10^6 and 10^7 cells s.c. into athymic nude mice. Within 3 to 4 weeks the ChH-1G cell line gave rise to tumors. No evidence of tumors was seen 4 months after injection with 10^7 ChH-Sp1 or ChH-7G3 cells.

**DISCUSSION**

These studies on the effects of naturally occurring modified purines on cell growth were not designed to test these compounds as transforming agents. The effect of the compounds on normal cells was unknown. The primary Chinese hamster embryo culture system was used because these cells have a finite life in culture, and thus any effects on growth might be more readily apparent than with established cell lines. In addition karyotype analysis of the cells is facilitated by the low number of chromosomes. We observed that chronic exposure of primary cultures to 1-methylguanine and 7-methylguanine leads to what is defined by the Tissue Culture Association as "in vitro transformation" (19). This is a "a heritable change occurring in cells in culture, resulting from exposure to chemicals, viruses, etc., leading to the acquisition of altered properties such as morphological, antigenic, neoplastic, proliferative, etc." (19). When the change is such that the cells can produce tumors in a compatible host, it is then termed "in vitro neoplastic (malignant) transformation." Freeman and Huebner (5) suggest that the expression of abnormal characteristics by cells other than cancer cells should be termed "genotype transformation."

Chinese hamster embryo cell cultures rarely give rise to continuous cell lines, and there are no published studies on the frequency of spontaneous conversion to continuous growth, although in cultures derived from adult differentiated cells it appears to be related to cell type (12). In our laboratory only 1 continuous line has developed spontaneously out of 10 untreated control cultures. If the 2 cultures exposed to guanine that did not convert to continuous lines are considered normal and are included in the calculation, then the spontaneous rate of conversion under the experimental conditions used is 8 to 10%.

All cultures exposed to 1-methylguanine and 7-methyl-

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**Table 1**

**Table 2**

**Table 1**

**Growth characteristics of Chinese hamster cells**

Saturation densities were determined after confluent cultures were split 1:2 and allowed to grow for 4 days. Duplicate cultures were trypsinized, and cells were counted with a hemacytometer. Growth in soft agar (0.33%) was established by the methods of MacPherson (14), with 10^* cells plated in 60-mm Petri dishes.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Primary culture</th>
<th>Passage</th>
<th>Saturation density (10^* cells/cm^2)</th>
<th>Growth in soft agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChH</td>
<td>V</td>
<td>4</td>
<td>0.4</td>
<td>–</td>
</tr>
<tr>
<td>ChH-Sp</td>
<td>II</td>
<td>85</td>
<td>1.4</td>
<td>–</td>
</tr>
<tr>
<td>ChH-1G</td>
<td>II</td>
<td>76</td>
<td>3.8</td>
<td>+</td>
</tr>
<tr>
<td>ChH-1G</td>
<td>II</td>
<td>92</td>
<td>4.3</td>
<td>+</td>
</tr>
<tr>
<td>Chh-1G</td>
<td>II</td>
<td>52</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>ChH-1G</td>
<td>III</td>
<td>43</td>
<td>1.2</td>
<td>–</td>
</tr>
<tr>
<td>ChH-1G</td>
<td>IV</td>
<td>40</td>
<td>1.8</td>
<td>–</td>
</tr>
<tr>
<td>ChH-1G</td>
<td>IV</td>
<td>40</td>
<td>1.3</td>
<td>–</td>
</tr>
<tr>
<td>ChH-1G</td>
<td>IV</td>
<td>81</td>
<td>1.6</td>
<td>–</td>
</tr>
<tr>
<td>ChH-7G</td>
<td>II</td>
<td>66</td>
<td>1.6</td>
<td>–</td>
</tr>
<tr>
<td>ChH-7G</td>
<td>III</td>
<td>39</td>
<td>1.2</td>
<td>–</td>
</tr>
<tr>
<td>ChH-7G</td>
<td>III</td>
<td>50</td>
<td>1.7</td>
<td>+</td>
</tr>
<tr>
<td>Chh-BP</td>
<td>IV</td>
<td>40</td>
<td>1.7</td>
<td>+</td>
</tr>
<tr>
<td>Chh:SV40</td>
<td>VI</td>
<td>76</td>
<td>3.1</td>
<td>+</td>
</tr>
</tbody>
</table>

* ChH-7G and ChH-7G6, derived from Primary Culture I were lost by contamination before they were characterized. They had been subcultured continuously for over 30 passages at the time they were lost.

† A cloning efficiency of greater than 10% was observed for all cultures scored as positive.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Passage</th>
<th>tRNA methyltransferase activity (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChH</td>
<td>4</td>
<td>3.13 ± 0.12</td>
</tr>
<tr>
<td>ChH-Sp</td>
<td>85</td>
<td>5.07 ± 0.12</td>
</tr>
<tr>
<td>ChH-1G</td>
<td>83</td>
<td>5.87 ± 0.76</td>
</tr>
<tr>
<td>ChH-1G</td>
<td>102</td>
<td>8.40 ± 1.24</td>
</tr>
<tr>
<td>ChH-1G</td>
<td>59</td>
<td>5.45 ± 0.05</td>
</tr>
<tr>
<td>ChH-1G</td>
<td>55</td>
<td>5.97 ± 0.57</td>
</tr>
<tr>
<td>ChH-1G</td>
<td>46</td>
<td>5.75 ± 1.35</td>
</tr>
<tr>
<td>ChH-1G</td>
<td>47</td>
<td>3.63 ± 0.42</td>
</tr>
<tr>
<td>ChH-7G</td>
<td>87</td>
<td>7.73 ± 0.54</td>
</tr>
<tr>
<td>ChH-7G</td>
<td>72</td>
<td>5.00 ± 0.82</td>
</tr>
<tr>
<td>ChH-7G</td>
<td>45</td>
<td>5.33 ± 0.34</td>
</tr>
<tr>
<td>ChH-7G</td>
<td>45</td>
<td>5.67 ± 0.55</td>
</tr>
<tr>
<td>ChH-BP</td>
<td>49</td>
<td>7.63 ± 1.53</td>
</tr>
<tr>
<td>ChH:SV40</td>
<td>77</td>
<td>11.07 ± 1.88</td>
</tr>
</tbody>
</table>

* Mean ± S.E.; 3 experiments.

† Significantly different from ChH cells at passage 4; p = 0.05.
guanine gave rise to continuous cell lines, which exhibited cytogenetic variation and a diverse range of phenotypes. Such diversity is common when cultured cells are transformed by carcinogenic agents (5, 12, 16), and the heritable changes induced may or may not include tumorigenic potency (5, 16). One cell line, ChH-1G2, is tumorigenic; consequently, neoplastic transformation has been demonstrated. It is also likely that other isolates will prove to be tumorigenic, since good correlation between growth in soft agar and tumorigenicity has been reported (4, 17). In some embryo culture systems it may take as long as 18 months of culturing after cell treatment to achieve full expression of the ability to grow in soft agar and to produce tumors (4).

The mode of action by which 1-methylguanine and 7-methylguanine transform ChH cells is unknown, but certain of the cell lines do share properties similar to those of ChH cells transformed by the carcinogenic hydrocarbon 3,4-benzopyrene or by the DNA tumor virus SV40. The methylated guanines exhibit no cytotoxicity towards ChH cells or any other cell lines tested when included in the growth medium at a concentration of 10 μM. They are probably not extensively altered metabolically, inasmuch as it has been shown that 7-methylguanine is given to rats 95% is excreted unchanged (3). However, it is possible that after prolonged exposure to the compounds in vitro trace metabolites might accumulate even though the medium is changed frequently.

The induction of alterations in morphology and growth characteristics in cells in culture by methylated guanine derivatives points to the possibility that modified purines may have a similar role in vivo. Certain modified adenine compounds (cytokinins) stimulate growth and differentiation in plants (6, 18). 1-Methyladenine has been shown to induce oocyte maturation in starfish (8) and gamete release in sea cucumbers (7). That the methylated guanines can be removed after the continuous cell lines are established without affecting growth characteristics or cell morphology argues against hormone-like effects. However, the increased tRNA methyltransferase activity in the continuous cell lines could elevate the endogenous level of methylated bases, and thereby vitiate the need for an exogenous source.

The physiological impact of elevated levels of modified bases in the blood and urine of cancer patients (20) requires investigation. This is being undertaken with animal model systems to determine whether the effects of 1-methylguanine and 7-methylguanine on normal cells in vitro have counterparts in vivo.

ACKNOWLEDGMENTS

We wish to thank Dr. J. M. Lehman for providing ChH and ChH:SV40 cultures and assistance, and T. D. Friedrich for assistance. We would also like to thank Pat Poche and Gail Thurman for expert technical assistance.

REFERENCES

Fig. 1. ChH-Sp₁, ChH₁G₂, and ChH-7G₃ cells at low (a, b, c) and high (d, e, f) cell densities. May-Grünwald-Giemsa stain, × 220.
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