Effects of Chemopreventive and Antitelomerase Agents on the Spontaneous Immortalization of Breast Epithelial Cells

Brittney-Shea Herbert, Angelique C. Wright, Christina M. Passons, Woodring E. Wright, Iqbal Unnisa Ali, Levy Kopelovich, Jerry W. Shay

Background: Activation of telomerase is an early event in the development of breast and other cancers that may lead to cell immortalization, a critical and rate-limiting step in cancer progression. Breast epithelial cells from women with Li–Fraumeni syndrome (LFS) immortalize spontaneously and reproducibly in culture. We, therefore, tested whether immortalization of these cells could be prevented by treating them with chemopreventive agents and by inhibiting telomerase activity. Methods: Nontoxic concentrations of the chemopreventive agents oltipraz, difluoromethylornithine, tamoxifen, and retinoic acid or with two different telomerase inhibitors. The frequency of spontaneous immortalization of LFS-derived cells was estimated by an approach based on fluctuation analyses. Statistical analyses were two-sided. Results: The frequency of spontaneous immortalization events of LFS-derived breast epithelial cells was reduced by long-term treatment with retinoic acid (P < .001) or tamoxifen (P < .05) compared with solvent-treated cells. The frequency of immortalization was also reduced by treating LFS-derived cells with an antitelomerase antisense oligonucleotide (P < .001) or by inducing the cells to express a dominant negative mutant of telomerase (P < .025) compared with cells treated with a control oligonucleotide or with empty vector, respectively. Conclusions: Treatment of preimmortal LFS breast epithelial cells with chemopreventive and antitelomerase agents decreased the frequency of spontaneous immortalization in vitro.

These studies validate the application of a new cell culture model system to screen the effects of novel chemopreventive agents by use of cell immortalization as an end point. The results also suggest that the telomerase ribonucleoprotein complex may be an important molecular target for breast cancer prevention. [J Natl Cancer Inst 2001;93:39–45]

Li–Fraumeni syndrome (LFS) is an autosomal dominant trait that results in childhood sarcomas and early-onset breast cancer. LFS is frequently characterized by inherited mutations in the tumor suppressor gene, p53 (also known as TP53) (1). Alterations in the p53 gene are thought to lead to genomic instability by allowing uncontrolled cellular proliferation, which perpetuates further instability (2). Breast tumors are the most prevalent cancer among women in LFS families; 25% of the breast cancers are diagnosed in women under age 30 years and 89% in women under age 50 years (1,3,4). The molecular mechanisms that lead to the specific increase in the incidence of breast cancer as opposed to other cancers in LFS families are not understood (5,6).

The cell immortalization that leads to breast cancer in LFS patients presumably involves the activation of telomerase. Telomerase is a ribonucleoprotein complex that adds telomeric repeats to the ends of chromosomes (7). In most normal human cells, with the exception of germ cells and stem cells, the chromosomes progressively shorten their telomeres with each cell division because telomerase is not active. Cellular senescence occurs when cells with critically shortened chromosomes undergo a permanent growth arrest.

Cell immortalization is thought to occur when the progressive telomere shortening that normally occurs in most cells is prevented by the reactivation or increased expression of telomerase, although other mechanisms that stabilize and maintain telomeres are also possible (8). Indeed, telomerase activity was detected in nearly all (90%) cancers tested (9,10). Immortal cells emerge by escaping crisis, which is a period of balanced cell growth and cell death followed by a decrease in the total number of surviving cells. If telomerase activation is necessary for cell immortalization, then telomerase may provide a target for cancer treatment and prevention. Previous reports (11–13) have demonstrated that treatment of immortal and cancerous cell lines with antitelomerase agents can inhibit their growth, leading to apoptotic cell death in a p53-independent manner.

To study the effects of chemopreventive and antitelomerase agents on immortalization, it is necessary to examine cells that become immortal in vitro. However, the spontaneous immortalization of human cells in culture is an extremely rare event: It requires mutations in several genes, such as p16, p53, and pRB, and their cellular pathways that are involved in cellular senescence (8,14,15). Although normal human breast epithelial cells in vitro never immortalize spontaneously, breast epithelial cells derived from LFS patients do so at a detectable and reproducible frequency of 5 × 10−7. These cells, which initially are heterozygous for wild-type p53 and lack telomerase activity, spontaneously inactivate p16 and the remaining wild-type p53 allele in vitro, resulting in immortalized cultures that express telomerase (16).

To investigate the nature of the immortalization process, we surveyed a panel of clinically validated chemopreventive agents for their effects on the spontaneous immortalization of LFS-derived breast epithelial cells in vitro. Because immortalization usually involves the activation of telomerase, we also used this model system to examine the effects of antitelomerase agents on breast epithelial cell immortalization. Telomerase activation occurs early in breast cancer progression; hence, it is an attractive target for the treatment and prevention of breast cancer (10,17–20). We examined two antitelomerase agents: B.-S. Herbert, A. C. Wright, C. M. Passons, W. E. Wright, J. W. Shay, Department of Cell Biology, The University of Texas Southwestern Medical Center, Dallas; I. U. Ali, L. Kopelovich, Division of Cancer Prevention, National Cancer Institute, Bethesda, MD.

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erase agents: One is an antisense oligonucleotide complementary to the template region of human telomerase RNA, and the other is a dominant-negative mutant of the catalytic subunit of human telomerase. These studies provide a new model to screen novel chemopreventive agents that target cellular immortalization, a critical step in cancer progression.

MATERIALS AND METHODS

Cell Culture

The human mammary epithelial (HME) cells used for these experiments were derived from the noncancerous breast tissue of a 31-year-old female diagnosed with LFS described previously (16). These cells contain a germline mutation (Met133Thr) in the p53 gene that affects the conformation of the wild-type p53 protein (16). Immunohistochemical analysis confirmed that these cells do not express the estrogen receptor-α (data not shown). The cells were grown in MCDB 170 media (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) supplemented with 0.4% bovine pituitary extract (Hammond Cell Tech, Alameda, CA), 10 ng/mL epidermal growth factor (Life Technologies, Inc.), and 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 5 μg/mL transferrin, and 50 μg/mL gentamicin (all from Sigma Chemical Co.; St. Louis, MO). The medium was changed every 2–3 days.

Treatment With Telomerase Inhibitors

Antitelomerase agents. Phosphorothioate-modified 2’-O-methyl RNA oligomers were purchased from Oligos Etc. (Wilsonville, OR). The sequence of the antisense RNA complementary to the template region of human telomerase RNA is 5’-CAGUUAAGGGUAAG-3’; the mismatched RNA is 5’-CAGUUAAGAAAGGAAG-3’, where the underlined nucleotides possess phosphorothioate linkages and mismatched nucleotides are indicated by italics. The antisense and mismatched oligomers were introduced into cells every 4 days during the course of the study by transfection with FuGENE6-transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN).

Construction and expression of dominant-negative telomerase mutant. The D869A-hTERT complementary DNA (cDNA) is a derivative of the modified hTERT cDNA that contains a point mutation within the highly conserved reverse transcriptase motif that changes the aspartic acid residue at amino acid 869 to an alanine (21). The D869A-hTERT cDNA was inserted into the EcoRI site of the retroviral vector pBABEpuro to create pBABEpuro D869A-hTERT (22). The pBABEpuro D869A-hTERT and pBABEpuro (empty vector) were introduced separately into the mouse ectropic packaging cell line PES01 (25) by electroporation. The ectropic viruses that were produced were supplemented with polybrene (Sigma Chemical Co.) to a final concentration of 4 μg/mL and used to infect the mouse amphotropic packaging cell line, PA317 (25). Infected cells were cultured in the presence of 4 μg/mL puromycin (Sigma Chemical Co.) for 1 week to select for puromycin-resistant cells that contained pBABEpuro D869A-hTERT or pBABEpuro (empty vector). The culture supernatants containing amphotropic viruses were then collected, supplemented with polybrene to a final concentration of 4 μg/mL, and used to infect HME cells. Infected cells were cultured in the presence of 150 ng/mL puromycin to select for puromycin-resistant cells that contained pBABEpuro D869A-hTERT or pBABEpuro (empty vector).

Treatment With Chemopreventive Agents

HME cells were treated every 3 days for approximately 25 population doublings with the following clinically validated chemopreventive agents: oltipraz, difluoromethylornithine (DFMO), tamoxifen citrate, 9-cis-retinoic acid, and 13-cis-retinoic acid. Oltipraz, DFMO, tamoxifen citrate, 9-cis-retinoic acid, and 13-cis-retinoic acid were obtained from the DCP Repository (McKesson BioServices, Rockville, MD). 13-cis-Retinoic acid was purchased from Sigma Chemical Co. Oltipraz, tamoxifen citrate, 9-cis-retinoic acid, and 13-cis-retinoic acid were dissolved in dimethyl sulfoxide and stored at −80 °C as 1000× stocks. DFMO was dissolved in HME culture media and stored at −80 °C as 1000× stocks. Chemopreventive agents were diluted to the appropriate working concentrations in HME culture media.

Fluctuation Analysis to Determine the Frequency of Immortalization

The frequency of immortalization was estimated by use of a fluctuation analysis as described previously (16,24,25). Each clone of cells that emerged from crisis was counted as an independent immortalization event. Frequency is defined as the probability of obtaining an immortal cell line on the basis of the total number of cells plated at each passage, not on the number of cell divisions, and is calculated by dividing the total number of independent immortalization events among all dishes by the total number of cells plated. In this study, untreated, mock-treated, and treated cells were expanded to 10 × 10^6 cells per dish. Once the cells reached crisis, they were harvested, replated, and re-treated at least once every 3 weeks until virtually no surviving cells remained or until the culture had a small focus of growing cells. Cells were considered to be immortal if they expressed telomerase and had undergone vigorous, postcrisis growth (16). Each small focus of growing cells was counted as an immortal event; the total number of immortal events from each of the 10 dishes for each treatment was recorded. Frequency of immortalization was calculated by dividing the number of immortal events by the total number of cells plated (10 × 10^6 cells). For each set of treatments, we performed and averaged three fluctuation analyses at three separate times by use of the same starting populations of cells recovered from freezer stocks.

Measurement of Telomerase Activity

Cells were resuspended in lysis buffer (i.e., 10 mM Tris–HCl [pH 8.0], 1 mM MgCl₂, 1 mM EGTA, 1% Nonidet P-40, 0.25 mM sodium deoxycholate, 10% glycerol, 150 mM NaCl, and 5 mM β-mercaptoethanol) at a concentration of 1000 cells/μL, were incubated on ice for 30 minutes, and were then centrifuged at 14 000g for 20 minutes at 4 °C (21,26). The resulting supernatants were used directly to detect telomerase activity (by use of 500 cell equivalents per assay) or were flash-frozen and stored at −80°C. Telomerase activity was measured by use of the TRAP-ene Telomerase Detection kit (Intergen, Purchase, NY), with the telomerase substrate (TS) primer (5’-AATCCGTCGAGCAGGTT-3’) as the substrate. After the extension of the substrate by telomerase (for 30 minutes at room temperature), the extension products were amplified by polymerase chain reaction by use of the TS primer and labeled with 32P, resolved on a 10% polyacrylamide gel, and revealed by exposure to a PhosphorImaging cassette (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis

Each dish was counted as having an immortalization event occur or not having an immortalization event occur. Because the data were collected as three different experiments for the cells treated with chemopreventive agents (Table 1) and with antitelomerase agents (Table 2) and the proportion of dishes with an immortal event were small in some treatments, a two-tailed Fisher’s exact test, using the number of dishes with an immortal event, was performed for each agent separately to examine the association between treatment and experiment to determine if the data across the three experiments for each treatment could be combined in further analyses. Because there was no statistically significant association between experiment and treatment for the chemopreventive agents (P = .932) or for the antitelomerase agents (P = .915), the data for each treatment across three experiments were combined. Comparisons of immortalization events for the seven chemopreventive treatments and the five antitelomerase treatments were performed separately by use of two-sided chi-square tests of independence and, when significant, were followed by Tukey-type post hoc multiple comparison tests for proportions (27) to examine which treatments were statistically significantly different. The alpha level for all statistical tests was set to 0.05 and the Fisher’s exact and chi-square analyses were performed by use of the SAS program (version 6.12; SAS Institute, Cary, NC). The Tukey-type post hoc multiple comparisons tests for proportions were programmed in Microsoft Excel, and significance was determined by consulting “Critical Values of the q Distribution” by Harter [reprinted in (27), Appendix p. 57–73], where exact P values are not available.

RESULTS

Concentrations of Chemopreventive Agents Used for Long-Term Study

To determine any cytotoxic effects of the chemopreventive agents, we tested a range of concentrations for each of the chemopreventive agents. Fig. 1, A, shows the dose–response of LFS-derived breast epithelial cells treated with 1 mM–100 μM of oltipraz, DFMO, tamoxifen, 9-cis-retinoic acid, and 13-cis-retinoic acid compared with that of control cells. The

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Untreated 5/10 5/10 6/10 5.33

Untreated 5/10 5/10 6/10 5.33

×

untreated control cells, having the lower proportion of immortalized cells, were compared with each of the other treated cells. The solvent-treated control cells, having the lower proportion of immortalization events compared with untreated or solvent-treated cells, were considered to be spontaneously immortalized because they continued to grow vigorously after crisis (16). The small fraction of cells that survived crisis expressed telomerase activity (Fig. 2, A) and were considered to be spontaneously immortalized.

Materials and Methods

©Materials and Methods©

The frequency of spontaneous immortalization is expressed as the probability of obtaining an immortal clone of cells emerging from crisis in a dish. Each dish was maintained at 10^6 cells/dish.

Table 1. Effects of chemopreventive agents on the spontaneous immortalization of Li-Fraumeni syndrome-derived breast epithelial cells

<table>
<thead>
<tr>
<th>Treatment, concentration*</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>P§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>5/10</td>
<td>5/10</td>
<td>6/10</td>
<td>5.33 \times 10^{-7}</td>
</tr>
<tr>
<td>Solvent</td>
<td>4/10</td>
<td>6/10</td>
<td>5/10</td>
<td>5.0 \times 10^{-7}</td>
</tr>
<tr>
<td>Olitipraz, 10 nM</td>
<td>3/10</td>
<td>3/10</td>
<td>4/10</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Diffuromethylanthine, 10 nM</td>
<td>3/10</td>
<td>3/10</td>
<td>1/10</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Tamoxifen citrate, 10 nM</td>
<td>2/10</td>
<td>1/10</td>
<td>1/10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>9-cis-Retinoic acid, 10 nM</td>
<td>2/10</td>
<td>0/10</td>
<td>0/10</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>13-cis-Retinoic acid, 1 nM</td>
<td>1/10</td>
<td>0/10</td>
<td>0/10</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Cells were treated for several months with nontoxic concentrations of chemopreventive agents (see the “Materials and Methods” section). Three separate experiments were conducted, and the numbers of immortalization events are shown for each.

†An immortalization event is expressed as a clone of cells emerging from crisis in a dish. Each dish was maintained at 10^6 cells/dish.

‡The combined data had a chi-square of 37.738, 6 df, and a P = .001.

§P values from the Harter tables [reprinted in (27)] from Tukey-type multiple-comparison tests for proportions comparing solvent-treated control cells. Untreated and solvent-treated controls were found to be not statistically significantly different. The solvent-treated control cells, having the lower proportion of immortalized cells, were compared with each of the other treated cells.

The frequency of spontaneous immortalization is expressed as the probability of obtaining an immortal cell line based on the total number of cells plated. For example, if one maintained 10 dishes at a minimum population size of 10^6 cells per dish, for a total pool size of 10 \times 10^6 (or 10^7), and three immortalization events in three dishes were observed, this would yield a frequency of 3 divided by 10^7, 3.0 \times 10^{-7} (16). Statistical analyses were performed using the number of immortalization events, not frequency.

Table 2. Effects of anti-telomerase agents on the spontaneous immortalization of Li-Fraumeni syndrome-derived breast epithelial cells

<table>
<thead>
<tr>
<th>Treatment, concentration*</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>P§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>5/10</td>
<td>5/10</td>
<td>6/10</td>
<td>5.33 \times 10^{-7}</td>
</tr>
<tr>
<td>Mismatch control, 500 nM</td>
<td>6/10</td>
<td>6/10</td>
<td>5/10</td>
<td>5.67 \times 10^{-7}</td>
</tr>
<tr>
<td>Antisense RNA, 500 nM</td>
<td>2/10</td>
<td>0/10</td>
<td>0/10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vector-negative hTERT</td>
<td>5/10</td>
<td>5/10</td>
<td>5/10</td>
<td>5.0 \times 10^{-7}</td>
</tr>
<tr>
<td>Dominant-negative hTERT</td>
<td>3/10</td>
<td>1/10</td>
<td>1/10</td>
<td>&lt;0.025</td>
</tr>
</tbody>
</table>

*Preimmortal cells were treated for several months with nontoxic concentrations of 2’-O-methyl RNA or mismatch oligonucleotides. A separate set of cells was also infected with D869A-hTERT, a dominant negative-acting mutant of hTERT several population doublings before crisis (see the “Materials and Methods” section). Three separate experiments were conducted, and the numbers of immortalization events are shown.

†An immortalization event is expressed as a clone of cells emerging from crisis in a dish. Each dish was maintained at 10^6 cells/dish.

‡The combined data had a chi-square of 27.847, 4 df, and a P = .001.

§P values from the Harter tables [reprinted in (27)] from Tukey-type multiple-comparison tests for proportions comparing untreated control cells. Untreated and mismatch oligonucleotide/vector-only treated controls were found to be not statistically significantly different. The untreated control cells, having the lower proportion of immortalized cells, were compared with each of the other antitelomerase-treated cells.

The frequency of spontaneous immortalization is expressed as the probability of obtaining an immortal cell line based on the total number of cells plated. For example, if one maintained 10 dishes at a minimum population size of 10^6 cells per dish, for a total pool size of 10 \times 10^6 (or 10^7), and three immortalization events in three dishes were observed, this would yield a frequency of 3 divided by 10^7, 3.0 \times 10^{-7} (16). Statistical analyses were performed using the number of immortalization events, not frequency.

Effect of Long-Term Treatment of LFS-Derived Breast Epithelial Cells With Antitelomerase Agents on the Frequency of Spontaneous Immortalization

To determine if clinically validated chemopreventive agents have any effect on cell immortalization, we treated preimmortal LFS breast epithelial cells before undergoing crisis with olitipraz, DFMO, retinoic acid, and tamoxifen. Before any treatment, all cells lacked telomerase activity. After the cells were grown for 25 population doublings in the presence or absence of the chemopreventive agents, they underwent a period of balanced growth and death (i.e., crisis) (16). The small fraction of cells that survived crisis expressed telomerase activity (Fig. 2, A) and were considered to be spontaneously immortalized because they continued to grow vigorously after crisis (16). Fluctuation analysis was used to estimate the frequency of immortalization for untreated and treated breast epithelial cells. As Table 1 shows, LFS-derived breast epithelial cells treated with tamoxifen, 9-cis-retinoic acid, and 13-cis-retinoic acid had fewer spontaneous immortalization events compared with untreated or solvent-treated cells (P<.05, P<.005, and P<.001 for tamoxifen, 9-cis-retinoic acid, and 13-cis-retinoic acid, respectively). Long-term treatment of the cells with olitipraz and DFMO also decreased the number of spontaneous immortalization events, but these effects were not statistically significant.

Effect of Long-Term Treatment of LFS-Derived Breast Epithelial Cells With Antitelomerase Agents on the Frequency of Spontaneous Immortalization

We treated LFS-derived breast epithelial cells with two different antitelomerase agents to determine if specifically inhibiting telomerase activity affected the frequency of spontaneous immortalization in these cells. One set of cells was transfected every 4 days over a 3-month period with an antisense RNA that was directed against the template region of human telomerase RNA. Another set of cells was infected with a retrovirus containing a dominant negative mutant of the telomerase catalytic subunit (hTERT) and then

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grown under conditions that selected for expression of the mutant protein. As summarized in Table 2, both antitelomerase agents decreased the absolute number of spontaneous immortalization events in the LFS-derived breast epithelial cells. The differences between untreated or mismatch- or vector-treated control cells and cells treated with the antisense oligonucleotide or the dominant-negative mutant hTERT were statistically significant ($P<.001$ and $P<.025$, respectively).

Because treatment with telomerase inhibitors did not completely prevent spontaneous immortalization of LFS-derived breast epithelial cells, we wanted to confirm whether telomerase was active in these cells after they had immortalized. The immortalized cultures derived from cells infected with the dominant-negative mutant of hTERT began to express telomerase activity, even though they were still resistant to the selectable drug resistance marker contained on the vector (data not shown). Western blot analysis detected no expression of the mutant hTERT in immortalized cells derived from cells infected with the dominant-negative mutant hTERT, confirming that the immortalized cells had lost the dominant-negative effect of the mutant hTERT because they no longer expressed the mutant protein (data not shown). The loss or decrease of expression of this mutant hTERT (and eventual reactivation of telomerase activity) within an infected cell population is not uncommon in tumor cell lines grown in continuous culture (13). Therefore, the appearance of immortalized clones from cells infected with the dominant-negative mutant of hTERT was most likely due to loss of expression of the mutant hTERT and reactivation of telomerase.

To ensure that the antitelomerase antisense oligonucleotide was an effective inhibitor of telomerase activity, we infected HME cells with a retrovirus containing wild-type hTERT (HME plus hTERT cells) and then transfected them with an antisense RNA complementary to the template region of human telomerase RNA. Three days after transfection, cells were collected and telomerase activity was measured. As shown in Fig. 2, B, transfection of HME plus hTERT cells with the antitelomerase antisense RNA effectively inhibited telomerase activity.

**DISCUSSION**

This is the first demonstration that nontoxic concentrations of chemopreventive and antitelomerase agents inhibit the spontaneous immortalization of human breast epithelial cells derived from an LFS patient. Our results suggest that this approach may be a useful model system to screen for chemopreventive agents that inhibit the progression of breast cancer. These results also support the use of antitelomerase agents as an approach to prevent the activation of telomerase.

Breast epithelial cells derived from an LFS patient provide an efficient model for examining the effects of treatment on the probability of immortalization because they spontaneously immortalize at a measurable frequency of $5 \times 10^{-7}$ [this study and (16)]. In contrast, most normal human cells, including breast epithelial cells, do not spontaneously immortalize at measurable frequencies in vitro (28,29).

Immortalization is thought to be a critical, rate-limiting step in cancer progression. One mechanism by which cells immortalize is by activating telomerase. The chemopreventive agents used in this study reduced the frequency of immortalization.
Telomerase activity was measured for 500 HME cell equivalents per lane by use of a radiolabeled oligonucleotide primer and a polymerase chain reaction (PCR)-based assay (see the “Materials and Methods” section). Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging. A ladder of bands represents the extension of the substrate primer by telomerase. Lane 1 = untreated HME cells that have spontaneously immortalized; lane 2 = preimmortal HME cells treated with oltipraz; lanes 3–5 = three independent immortalization events of HME cells treated with oltipraz; lane 6 = preimmortal HME cells treated with difluoromethylornithine (DFMO); lane 7 = one immortalization event of HME cells treated with DFMO; lane 8 = preimmortal HME cells treated with tamoxifen; lane 9 = one immortalization event of HME cells treated with tamoxifen; and lane 10 = preimmortal HME cells treated with retinoic acid. In this experiment, no immortalization events were observed in HME cells treated with retinoic acid. TAM = tamoxifen; RA = 9-cis retinoic acid.

Materials and Methods

Preimmortal HME cells derived from a patient with Li-Fraumeni syndrome were infected with a retrovirus containing wild-type hTERT to induce the cells to express telomerase activity. Preimmortal HME cells treated with retinoic acid. TAM = tamoxifen; RA = 9-cis retinoic acid. Panel B: Antisense RNA inhibits telomerase activity. Preimmortal HME cells derived from a patient with Li-Fraumeni syndrome were infected with a retrovirus containing wild-type hTERT to induce the cells to express telomerase activity. Cells expressing exogenous hTERT were then transfected with antisense RNA complementary to the template region of human telomerase RNA. Three days after transfection, cells were collected and telomerase activity was measured for 500-cell equivalents per lane by use of a radiolabeled oligonucleotide primer and a PCR-based assay (see the “Materials and Methods” section). Labeled PCR reaction products were resolved on polyacrylamide gels and visualized by PhosphorImaging. A ladder of bands represents the extension of the substrate primer by telomerase. Lane 1 = 500 H1299 lung carcinoma cells served as a positive control for the telomerase assay; lane 2 = lysis buffer served as a negative control; lane 3 = preimmortal HME cells; lane 4 = preimmortal HME cells infected with the hTERT-containing retrovirus; and lane 5 = preimmortal HME cells infected with the hTERT-containing retrovirus and treated with an antisense RNA oligonucleotide complementary to the template region of human telomerase RNA.

and, by some mechanism, must have prevented the ability of cells to emerge from crisis. In fact, a decrease in the frequency of immortalization was also observed for cells treated with chemopreventive agents for shorter times (only approximately 10 population doublings) before crisis. Moreover, removal of the treatments before crisis results in an immortalization frequency similar to that of untreated or mock-treated cells. These results suggest that the agents are likely to be affecting cells when they are in crisis. Whether these chemopreventive agents affect immortalization through prevention of telomerase activation is a current area of interest in our laboratory.

Since 90% of cancers exhibit telomerase activity and activation of telomerase is an early event in breast cancer progression, the development of chemoprevention strategies that specifically target telomerase is of great interest. The recent cloning of the hTERT promoter has enabled researchers to dissect the molecular mechanisms regulating transcription of hTERT (30,31). Kyo et al. (32) showed that estrogen increases hTERT messenger RNA and activates telomerase by inducing the binding of the estrogen receptor to estrogen-response elements on the hTERT promoter. Antiestrogen therapies may, therefore, directly or indirectly affect the regulation of telomerase expression and thus prevent a step that is necessary for the progression of most cancers. One such antiestrogen drug, tamoxifen, has recently been shown to affect telomerase activity and the proliferation of breast carcinoma cell lines (33). Although our results show that tamoxifen is a potent agent in the prevention of spontaneous immortalization, this effect is unlikely to involve antiestrogen activity because the HME cells used in this study do not contain the normal estrogen receptor. Tamoxifen has been shown, in some cases, to act independently of the estrogen receptor (34). Whether the observed effects of tamoxifen on spontaneous immortalization of the LFS cells were indirect (i.e., through an estrogen-like nuclear receptor) remains to be determined.

Retinoids also have been shown to reduce telomerase activity, as seen after the induction of differentiation by retinoic acid and a sharp decline in cell proliferation caused by the treatment of mammary tumors with the synthetic retinoid 4-(hydroxyphenyl) retinamide (35–38). These findings confirm the need to understand the molecular mechanisms by which conventional chemopreventive agents act via their respective receptors and to investigate whether they act by preventing the activation of telomerase during the progression to cancer.

Oltipraz and DFMO also decreased the frequency of immortalization, but not as much as the other chemopreventive agents used in this study. Oltipraz is an anticarcinogen that inhibits HIV-1 replication and some reverse transcriptases (39). The chemopreventive properties of oltipraz have been studied in animal models of colon and lung carcinogenesis (40,41). DFMO is a potent and irreversible inhibitor of the enzyme ornithine decarboxylase, which is involved in polyamine synthesis. DFMO has been reported to have chemopreventive activity in colon, skin, and breast carcinogenesis (42,43). Because polyamine synthesis is necessary for cellular proliferation, DFMO may derive its chemopreventive activity from its antiproliferative properties.

Although some of the chemopreventive agents may act indirectly on telomerase, other agents are being designed specifically to target telomerase directly (11–13,18,44–46). Oligonucleotides complementary to the template region of the RNA component of telomerase have been shown to inhibit telomerase activity and cell growth after long-term treatment of immortal and cancerous cell lines.
(11,47,48). The other telomerase inhibitor used in this study is a cDNA that contains a point mutation in one of the conserved reverse transcriptase motifs in the human telomerase catalytic subunit, hTERT. When assembled into the telomerase ribonucleoprotein complex, this mutant subunit acts in a dominant-negative fashion to inhibit telomerase activity in tumor cell lines, shortening telomeres and inducing cell death (12,13,21). Our study confirms the potential of anti telomerase agents to prevent the activation of telomerase and to inhibit spontaneous immortalization in vitro.

Our finding that treating cells before crisis with antitelomerase agents diminishes the spontaneous immortalization of LFS-derived breast epithelial cells in vitro indicates a potential approach for the development of rational chemoprevention strategies by use of both clinically and preclinically validated chemopreventive agents for women with a genetic predisposition to breast cancer. Moreover, the prevention of spontaneous immortalization described in this work offers a new intermediate end point for validating novel chemopreventive agents.

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NOTES

W. E. Wright and J. W. Shay are on the scientific advisory board and own stock in the Geron Corporation, Menlo Park, CA.

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