Suppression of cell proliferation and telomerase activity in 4-(hydroxyphenyl)retinamide-treated mammary tumors

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The detection of telomerase activity has been proposed as a biomarker of breast cancer development and progression. In this study, we used cell proliferation and telomerase in MNU (N-methyl-N-nitrosourea)-induced mammary carcinomas as targets for assessing the response of tumor cells to 4-(hydroxyphenyl)retinamide (4-HPR), a known inhibitor of mammary carcinogenesis in animal models and premenopausal women. In mammary tumors of rats treated for 1, 2, 4 or 6 weeks with 4-HPR, we observed that telomerase activity decreased progressively with the extension of 4-HPR administration. A marked reduction in telomerase activity was already observed by 2 weeks after treatment and the lowest level was found at 6 weeks after initiation of 4-HPR treatment. The changes in telomerase activity were preceded and accompanied by a significant decrease in the percentage of proliferating cells as evaluated by 5-bromodeoxyuridine (BrdU)-labeling. However, when the values of telomerase activity in the individual tumors were compared with the percentage of proliferating cells, no significant correlation was found. These data suggest that the decreased telomerase activity in the animals treated with 4-HPR is not a simple consequence of the changes in cell proliferation, but a more complex phenomenon involving different cellular mechanisms and pathways. The time-dependent and consistent decrease of telomerase activity in the tumors treated with 4-HPR suggests that, in addition to the percentage of proliferating cells, telomerase activity could also be used as an endpoint in breast cancer chemotherapy studies.

Introduction

Over the last few years, information has been accumulated on the role of telomerase in tumor development and progression (1–3). High telomerase activity was observed in practically all human breast cancers, and we and others detected significant activity already at the level of breast carcinoma in situ (4,5). Very recently, expression of the telomerase protein component (TERT) in normal breast epithelium was demonstrated by in situ hybridization (6). It was further shown that the levels and number of cells expressing telomerase increased during mammary carcinogenesis (6). Similarly, we recently demonstrated constitutive expression of telomerase in estrogen-regulated rat tissues, including the mammary epithelium, indicating that the telomerase-expressing cells belong to the stem cell compartment. Furthermore, we showed that telomerase does not associate with proliferation per se, but rather with proliferative potential (7). An increase in the levels of telomerase activity has also been described in rat mammary carcinomas (8).

Little is known about how various internal and external factors might affect telomerase activity in breast cells. It has been shown that telomerase activity is dramatically down-regulated in terminally differentiated cell populations (9,10). In vitro studies on human breast cancer cell lines showed that doxorubicin, cisplatinum and tomoxotomolide decreased telomerase activity, and this was dose- and time-dependent (11). Cells resistant to doxorubicin and tomoxotomolide showed no decline in telomerase activity and cell growth. Recently, in one clinical study, a limited number (n = 25) of patients with advanced breast carcinomas treated with chemotherapy showed a significant decrease in telomerase activity in their tumors when compared with untreated controls (12). These data suggest that the levels of telomerase activity in breast carcinomas could be used as a potential marker for assessing the effect of anti-neoplasic agents or of specific inhibitors of telomerase activity. Previous studies showed that 4-(hydroxyphenyl)retinamide (4-HPR) is a potent inhibitor of mammary carcinogenesis in animal models (13,14). It was also found that 4-HPR can induce regression and retardation of the growth of N-methyl-N-nitrosourea (MNU)-induced mammary carcinomas in rats (15). Very recently, Bischoff et al. (16) reported that targretin, a retinoid that is a retinoid X-receptor ligand, can also suppress the growth of established mammary carcinomas. These studies open a new avenue for potential implementation of retinoids not only for chemoprevention, but also for treatment of breast cancer. So far, no data have been published about whether 4-HPR or other retinoids can influence the levels of telomerase activity in mammary carcinomas, and whether this might correlate with changes in cell proliferation. Here, we report that 4-HPR dramatically suppresses cell proliferation and telomerase activity in MNU-induced mammary carcinomas, and that the longer the administration of 4-HPR, the lower the values of telomerase activity.

Materials and methods

Animals

Female, virgin Sprague–Dawley [Hsp. (SD/BR)] rats were obtained from Harlan Sprague–Dawley (Indianapolis, IN) at 35 days of age, and after 1 week of quarantine were randomized by weight and injected i.p. twice with MNU, at the ages of 43 and 50 days. The animals were fed 4% Purina Chow AIN–76A diet (Teklad, Madison, WI) ad libitum and had free access to water. The weight of the animals was measured once a week.

Chemical carcinogen

MNU was obtained from Ash Stevens Inc. (Detroit, MI), dissolved in sterile acidified saline (pH 5.0) and injected i.p. at dose of 50 mg/kg body wt as

Abbreviations: 4-HPR, 4-(hydroxyphenyl)retinamide; BrdU, 5-bromodeoxyuridine; ITAS, internal standard; MNU, N-methyl-N-nitrosourea; TERT, telomerase protein component; TRAP, telomere repeat amplification protocol.

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indicated above. Two doses of MNU were used in order to increase the number of tumors.

4-(Hydroxyphenyl)retinamide

4-HPR was obtained from R.W. Johnson Pharmaceutical Research Institute (Spring House, PA) and was added to the diet at 782 mg/kg (2 mM) diet. Animals with palpable (4–8 mm) mammary tumors were randomized and treated for different time intervals (1, 2, 4 and 6 weeks) with 4-HPR. Placebo diet containing the 4-HPR vehicle only was given to the control animals.

**Tumor volume**

Tumors were measured twice weekly, and tumor volume \(V \text{ mm}^3\) was calculated by the values of length \(L\), width \(W\) and thickness \(T\) of the tumors:

\[
V = \pi/6 \times L \times W \times T \text{ (mm}^3\text{)}
\]

The animals were killed by CO₂ narcosis, and tumors were removed and cut into two halves. One part of the tumor was fixed in 10% neutral formalin for histomorphology and immunocytochemistry, and the other part was frozen in liquid nitrogen for assessment of telomerase activity.

**Cell proliferation**

Proliferating cells in mammary carcinomas were labeled by 5-bromodeoxyuridine (BrdU, 50 mg/kg body wt; Sigma, St Louis, MO). The animals were killed 2 h after i.p. administration of BrdU. The nuclei labeled with BrdU were identified by an anti-BrdU monoclonal antibody (Beckton Dickinson, Palo Alto, CA) and ABC kit (Vector, Burlingame, CA), as described earlier (17). More than 1000 randomly distributed cells were scored from each tumor and the percentage of BrdU-labeled cells was expressed as BrdU-LI.

**Telomerase activity assay**

For telomerase detection, we used the PCR-mediated telomere repeat amplification protocol (TRAP) (18). As a positive control, an extract from cells with known telomerase activity (human breast cancer line MDA-MB-157, 100 cells equivalent) was used. As a negative control, cell extract was substituted for lysis buffer. As an additional control for the TRAP assay, we used an internal standard (ITAS; a gift of Dr J. Shay) that amplifies from the same primers (18). This internal standard, which consists of a 150 bp DNA product, allows identification of false-negative tumor samples that might contain Taq polymerase inhibitors. Cell extracts were obtained and TRAP was performed as previously described (18) with minor modifications (19). Two microliters of tissue extract (protein concentration 0.5 μg/μl) were used per assay. The CX primer, ITAS and Taq DNA polymerase (7 U per assay) were added to each sample after 5 min incubation at 90.8°C to make a hot start. Aliquots (10 μl) of the PCR mixture were analysed on 0.4 mm, 8% non-denaturing acrylamide gels (20×40 cm), run in 0.5×TBE buffer until the xylene cyanol had migrated 17 cm from the origin. The gels were then dried and exposed for 20 h to hyperfilm MP films (Amersham, Arlington Heights, IL). Following autoradiography, gels were also analysed after overnight exposure with a Molecular Dynamics PhosphorImager (Sunnyvale, CA). The area of integration of all peaks was normalized to the signal from the internal standard, then, after background subtraction, expressed relative to the positive control signal (100 cell equivalent) that was run with each experiment. The method described is only semi-quantitative, but it is sufficient for the comparative analysis of tumors relative to the same positive control cell extract.

**Statistical analysis**

The comparison of mean values between the different groups was evaluated by ANOVA with Fisher’s LSD test.

**Results**

4-HPR suppresses the growth of established mammary carcinomas

The changes in tumor volume in control and 4-HPR-treated animals are presented in Figure 1. 4-HPR treatment was initiated when the tumors were small in size (4–8 mm). Two weeks of treatment with 4-HPR were not sufficient to suppress tumor growth. However, with extension of 4-HPR administration (4–6 weeks), the growth potential of tumors decreased compared with those in control animals. Among the animals treated with 4-HPR a substantial variability in tumor volume was observed with a significant decrease (\(P < 0.01\)) in the animals killed 6 weeks after initiation of 4-HPR administration only (Table I). The weight of the animals remained close for all time points with no statistical difference (\(P > 0.05\)) between control and 4-HPR treated groups (Table I), indicating that 4-HPR at 2 mM/kg diet is not toxic and does not affect growth.

4-HPR inhibits cell proliferation in mammary carcinomas

BrdU-labeled cells in the control tumors were either randomly distributed among the tumor parenchyma or were located close to the stroma (proliferative zone, Figure 2a). Viable tumor cells lacking proliferating activity were frequently identified between the proliferating peripheral zones and the central necrosis. In the animals treated with 4-HPR, an overall reduction in proliferating cells was found (Figure 2b). However, myoepithelial and stroma cells continued to proliferate, indicating that 4-HPR inhibits mostly the epithelial cell proliferation. Among tumor parenchyma, there were areas lacking BrdU-labeled cells, as well as areas with a high number of labeled cells, suggesting that tumor cells are differentially sensitive to the antineoplastic effect of 4-HPR. In the control tumors, BrdU-LI was in the range between 15 and 38% with a mean value of 26.2 ± 6.2%, whereas in the animals treated with 4-HPR a significant decrease in BrdU-LI for all time points examined (1, 2, 4 and 6 weeks of treatment) was found. The differences in BrdU-LI values between various time points were insignificant (\(P > 0.05\)). In most of the animals treated with 4-HPR a significant variability in BrdU-LI values was observed. For instance, in the animals treated for 2 weeks with 4-HPR there were tumors with BrdU-LI below 1%, as well as above 15% (Figure 3a). In most tumors 4-HPR suppressed cell proliferation in the peripheral tumor areas where tissue disintegration was also observed (Figure 2b).

4-HPR causes a progressive decline in telomerase activity in mammary tumors

Telomerase activity as evaluated by the TRAP assay was fairly variable in tumors from control untreated rats. A uniform suppression of telomerase activity was observed paralleling the extent of 4-HPR treatment. By 2 weeks of treatment, a reduction in telomerase activity was evident in most tumors analysed and the lowest level of telomerase activity was found.
Inhibition of mammary carcinogenesis with retinoids

Table I. Effects of 4-HPR on body size weights

<table>
<thead>
<tr>
<th>Treatment (weeks)</th>
<th>MNU</th>
<th>MNU + 4-HPR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Body wt (g)</td>
</tr>
<tr>
<td>0</td>
<td>10⁵</td>
<td>228 ± 10</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>231 ± 12</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>240 ± 17</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>247 ± 18</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>50 ± 25</td>
</tr>
</tbody>
</table>

*Ten control animals with mammary carcinomas were followed for 6 weeks, and their body weight (g) and tumor size (mm³) were monitored. The animals were killed, and tumors were used for assessment of cell proliferation and telomerase activity.

*bTotal number of animals treated with 4-HPR. These animals were separated into four groups and killed 1, 2, 4 and 6 weeks after initiation of 4-HPR administration. At the time of death the body weight (g) and tumor size (mm³) were assessed.

*cIndicates significant differences in the size of control and 4-HPR treated tumors (P < 0.01).

Fig. 2. (a) A high number of BrdU-labeled cells in a control tumor with histology of adenocarcinoma. Most BrdU-labeled cells are located close to the basal membrane. The slide is counter-stained by haematoxylin, ×200. (b) A decrease in the number of BrdU-labeled tumor cells in animals treated for 2 weeks with BrdU. The tumor is adenocarcinoma and has histology similar to those of control tumor (a). There are areas of tissue disintegration (arrows). The slide is counter-stained by haematoxylin, ×200.

at 6 weeks after initiation of 4-HPR treatment with little variability in telomerase levels between tumors (Figures 3b and 4). Although telomerase activity decreased concomitant with inhibition of cell proliferation, we did not observe a statistically significant direct correlation with the levels of telomerase activity and cell proliferation analysed by non-parametric Spearman rank correlation. BrdU-LI data (see below), when compared with telomerase activity, suggest that 4-HPR initially inhibits cell proliferation and later decreases telomerase activity, since, by 1 week of 4-HPR treatment,
BrdU-LI was significantly lower than the control values, although telomerase levels in most of the tumors was still high.

Discussion

In this study, we assessed the anti-tumor effect of 4-HPR on MNU-induced mammary carcinomas. Endpoint biomarkers were tumor volume, cell proliferation and telomerase activity. In most previous studies (except one, ref. 15), 4-HPR has been used as an inhibitor of mammary carcinogenesis (i.e. chemopreventive), but not of tumor growth (14). We observed that 4-HPR, when given in the diet of animals with established mammary carcinomas, suppresses cell proliferation and telomerase activity. To the best of our knowledge, this is the first study reporting the effect of 4-HPR on cell proliferation and telomerase activity in established mammary carcinomas in rats. In a recent study, which is still in progress, we observed that, in the animals treated with 4-HPR, telomerase activity decreased in a time-dependent fashion, with the lowest values 6 weeks after initiation of treatment, when the experiment was terminated. It appears that the decrease in telomerase activity is preceded and accompanied by decrease in cell proliferation. However, the increase in cell proliferation in some tumors in the animals killed 4 and 6 weeks after initiation of treatment with 4-HPR (when telomerase activity in the tumors remained low), and lack of strong correlation between the values of BrdU-LI and telomerase activity in individual tumors suggest that other factors, in addition to cell proliferation, are associated with telomerase activity regulation.

It has been reported that telomerase is variably expressed in MNU-induced mammary carcinomas (8). Here, we observed that the heterogeneity in telomerase activity was still very significant within 1 week of treatment, but was reduced after 6 weeks of 4-HPR administration, with all tumors showing low levels of telomerase activity (Figure 3b). These data suggest that 4-HPR causes a marked decline in the number of telomerase-expressing cells and, perhaps, in the levels of expression, bringing the enzyme activity to a baseline level that seems to be similar in all treated tumors. The half life of telomerase is apparently quite long (22) and this is important, considering that the decrease in activity we observed was slow and progressive, rather than abrupt, which would be expected if cell death is the major mechanism of eliminating telomerase-expressing cells (11). It is possible to speculate that 4-HPR predominantly eliminates tumor cell subpopulations exhibiting high proliferative potential and high levels of telomerase expression, and only cells with low residual activity remained after treatment. Using in vitro systems, other investigators have previously demonstrated a pronounced down-regulation of telomerase activity as a consequence of induction of differentiation by retinoic acid (9,10,23). It has also been shown that retinoids induce morphological and functional differentiation of normal rat mammary epithelial cells (24). Here, we demonstrate that retinoids in established mammary carcinomas also cause a marked decline in telomerase activity, which is preceded by a sharp decrease in cell proliferation. This allows speculation that, in addition to a possible selective killing of cells with high proliferative potential, the decrease in telomerase activity observed in tumors from 4-HPR treated animals may also be the consequence of induction of cellular differentiation and withdrawal from the pool of proliferating cells.

In conclusion, the results obtained in this study suggest that, in addition to cell proliferation, telomerase activity could also be used as a potential endpoint biomarker in assessing the effect of 4-HPR and probably other retinoids on experimental and possibly human breast cancer.

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References

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