Evidence for UV-associated Activation of Telomerase in Human Skin

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Abstract

Telomerase activation plays a crucial role in the immortalization of human cells and carcinogenesis; however, the temporal and pathophysiological aspects of the activation in vivo are poorly understood. We found telomerase activity not only in malignant tumors (91%) but also in most benign (60%) and premalignant (89%) skin tumors. This suggests the involvement of telomerase activation in a crucial biological step of human skin carcinogenesis. Because UV light is a major factor in skin carcinogenesis, we further examined telomerase activity in normal skin samples and in normal skin samples adjacent to benign, premalignant, and malignant skin lesions. Data for chronically sun-exposed body sites were compared with those for covered sites. Among normal skin samples, 39% (26 of 67) had telomerase activity, and this activity was unrelated to neighboring lesions but strongly associated with the level of sun exposure. Fifty-four% (21 of 39) of normal skin samples from chronically sun-exposed sites were telomerase-positive, compared with only 12% (3 of 26) of samples from covered sites. When we examined telomerase activity and CC to TT mutations at codons 247/8 of the p53 gene (which are considered to be UV specific) in the same normal skin samples, only 43% (7 of 16) of telomerase-positive normal skin samples at sun-exposed sites contained the p53 mutations, whereas all (7 of 7) of the samples with UV-specific p53 mutations showed telomerase activity ($P = 0.019$). These data suggest that telomerase activation is involved at an early stage of human skin carcinogenesis and that activation may precede the acquisition of UV-associated p53 mutations in the skin. Telomerase activity was also found in plucked hair follicles and enzymatically separated epidermis, which may be associated with the presence of stem cells in the skin.

Introduction

Skin cancer is the most prevalent human cancer, and solar UV radiation is its major etiological factor (1). The precise molecular events involved in multistage skin carcinogenesis are not yet fully understood. p53 gene mutations are the most frequent genetic alterations found in human skin tumors thus far (2, 3). We have previously shown that a substantial proportion of cells in morphologically normal skin of Australian skin cancer patients already have UV-specific CC to TT p53 gene mutations (4).

Inactivation of two tumor suppressor genes, p53 and retinoblastoma, is suspected to be a common molecular pathway for human keratinocyte immortalization in vitro. However, the molecular steps in vivo leading to such inactivation and from that inactivation to the most prevalent human tumor, nonmelanocytic skin cancer, have not been identified. As has been proposed for tumors of various organs, skin cancer usually develops through a multistage pathway accompanied by various genetic alterations (5). Telomerase is a ribonucleoprotein enzyme (a RNA-dependent DNA polymerase) that catalyzes addition of telomeric repeats (TTAGGG), to telomeric DNA termini using a segment of its endogenous RNA component as a template (6, 7). It has been postulated that without telomerase, somatic cells cannot divide beyond their limited life span due to accumulation of chromosome abnormalities accompanied by mitotic failure. The development of a sensitive telomerase activity assay, TRAP (8), revealed that in approximately 90% of more than 1000 human tumor biopsies (8, 9), telomerase was activated. However, the enzyme was not activated in normal adult somatic tissue samples, with the exception of embryonic tissues, such as the germline, testis, and ovary, and in renewal tissues, such as pluripotent blood cells (leukocytes and T-cells) and endometrium (8, 10–14).

Various in vitro studies and tumor analyses have led to the conclusion that activation of telomerase is the most universal biological pathway in human carcinogenesis (8, 10, 15–17). In addition to being active in malignant tumors, telomerase is already activated in various benign or premalignant lesions of gastric cancer (gastric intestinal metaplasia), colon cancer (colorectal tubular adenoma; Ref. 18), and meningioma (benign meningiomas). It is interesting, therefore, to study a possible correlation between telomerase activity, UV exposure, and p53 gene mutations.

In this paper, we have (a) assessed the temporal aspects of telomerase activation in human skin carcinogenesis by examining the telomerase activity in premalignant skin lesions of various pathologies and at various stages of tumor progression, in normal skin biopsies from patients without skin cancer, and in normal skin biopsies adjacent to the skin lesions in relation to chronic sun exposure; (b) elucidated the molecular pathway of UV-associated skin carcinogenesis by comparing telomerase activity with the acquisition of UV-specific p53 mutations in the same skin biopsies in relation to sun exposure; and (c) localized the activity in normal skin and attempted to identify the cell population having telomerase activity in the skin (perhaps in stem cells) by examining the activity in enzymatically separated epidermis and plucked hair follicles.

Materials and Methods

Skin Biopsy Samples

Portions of surgically excised skin tumors and normal skin biopsies were carefully dissected, snap frozen in liquid nitrogen immediately after excision, and stored at −80°C until telomerase and DNA extraction were carried out. The remaining portions were used for pathological examination, counting of lymphoid cell infiltration around the tumors, and DNA extraction.

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3 The abbreviations used are: TRAP, telomere repeat amplification protocol; AK, actinic keratosis; BD, Bowen's disease; MM, malignant melanoma; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; XP, xeroderma pigmentosum.


370
UV-ASSOCIATED TELOMERASE ACTIVITY IN THE SKIN

Skin Biopsies. Thirty-three samples of normal skin from Japanese and Caucasian patients without a skin tumor, 6 normal skin samples distant from premalignant tumors or skin cancers, 8 normal skin samples adjacent to benign skin tumors, such as seborrheic keratosis and nevus cell nevus, and 30 normal skin samples adjacent to premalignant tumors and skin cancers were analyzed. The skin tumors examined were as follows: (a) benign tumors (4 seborrheic keratosis and 1 eccrine poroma); (b) premalignant tumors (9 AKs, 5 BDs, 1 MM in situ, 2 genital Paget’s disease, and 1 sebaceous epithelioma); (c) primary skin cancers (13 BCCs, 6 SCCs, 5 MMs, and 1 eccrine carcinoma). One AK and one MM were derived from a Japanese patient, and a BCC was derived from another Japanese patient (complementation groups were not assigned). Thirty-eight tumor samples were obtained from Japanese patients, and 4 SCCs, 6 BCCs, 2 primary MMs, and 1 AK were obtained from Caucasian patients. Histological analysis revealed a slight to moderate lymphoid cell infiltrate around skin tumors in most cases, but little infiltration was usually seen in adjacent normal skin. Adjacent (microscopically) normal skin biopsies were usually at least 5 mm away from the edge of the respective skin lesions. Epidermis was separated from some samples by trypsin (Life Technologies, Inc.) treatment overnight at 4°C as described previously (4).

Hair Follicles. Five anagen hairs (exclamation hair) at the growth stage of the hair cycle from the scalp of each of 22 volunteers (11 Japanese males, 2 Caucasian females, and 9 Caucasian males) were plucked, and a portion of the hair follicle was subjected to TRAP assay. Some of the hair follicles (the portion covered with a gelatinous capsule) were carefully dissected by razor blade under a dissection microscope into three parts before the TRAP assay (Fig. 1C, ii): the upper part of the hair follicle covered with gelatinous capsule and a part of the hair shaft, the middle part of the hair follicle, beside the hair bulge, and the lower part of the hair follicle, including the hair bulb.

Telomerase Activity Assay

Each frozen skin sample was carefully reexamined morphologically and dissected to a size of approximately 100 mm² before telomerase extraction. Samples for a TRAP telomerase activity assay were briefly washed with 1 ml of washing buffer for 5 min on ice as described (8). After the washing buffer was removed, 100 µl of ice-cold lysis buffer was added. The samples were homogenized three times for 1 min each on ice with a plastic pestle and kept on ice for 30 min. The lysates were then centrifuged at 15,000 rpm for 30 min at 4°C, and the supernatant was stored at −80°C until use. A part of the lysate (approximately 3–5 µg of protein) was used for the TRAP assay as described by Kim et al. (8). An aliquot of the PCR products (15 of 50 µl) was separated by electrophoresis on an 8% polyacrylamide gel (19:1) for 2 h at 200 V, and the gel was then dried, exposed for 2–3 h, and analyzed by a PhosphorImager (Molecular Dynamics).

Detection of CC to TT Mutations of the p53 Gene in Normal Skin Samples

DNA was extracted from the same biopsies that were tested for telomerase activity, and 5 µg of the extracted DNA were subjected to the p53 mutant frequency assay (mutant allele-specific PCR reaction) as described previously (4). The PCR products (30 µl) were separated by electrophoresis on an 8% polyacrylamide gel (19:1). The gel was then dried and exposed for 2–3 h, and the UV-specific CC to TT p53 mutation (at codon 247/8) was detected and evaluated by a PhosphorImager (Molecular Dynamics) with scanner control and ImageQuant, version 1.1 (Molecular Dynamics).

Statistical Analysis

Fisher’s probability test and the χ² test were performed with StatView (version 4.51, ABACUS).

Results

Telomerase Activity in Benign and Premalignant Skin Tumors. In three of five benign tumors (seborrheic keratosis) occurring at covered sites (60%), telomerase was positive. Sixteen of 18 premalignant skin tumors were telomerase positive, irrespective of the anatomical site (at sun-exposed sites, 100% (12 of 12); at covered sites, 67% (4 of 6)). Among primary tumors, 11 of 13 BCCs (at sun-exposed sites, 80% (8 of 10); at covered sites, 100% (3 of 3)), 6 of 6 SCCs (at sun-exposed sites, 100% (4 of 4); at covered sites 100% (2 of 2)), 4 of 5 MMs (at sun-exposed sites, 100% (1 of 1); at covered sites, 75% (3 of 4)), and 1 eccrine carcinoma (at sun-exposed site, 100% (1 of 1)) showed telomerase activity. Because UV light is a major etiological factor in human skin carcinogenesis (1, 3), various skin tumor biopsy samples from commonly sun-exposed sites (face and back of hands) were compared with samples from covered sites.

Telomerase Activity in Normal Skin and Normal Skin Adjacent to Skin Tumors. Sixty-five normal skin biopsies from patients with or without skin tumors were analyzed, as shown in Fig. 1A. “Normal” skin adjacent to skin tumors was at least 5 mm away from the lesions and free of tumor cells as determined by histological examination. In total, 24 of 65 (37%; 13 of 37 (35%) in normal skin and 11 of 28 (39%) in normal skin adjacent to the skin lesions) normal skin samples were telomerase positive (Table 1).

Association of Telomerase Activity with Chronic Sun Exposure. In skin tumors, incidence of telomerase activity generally has no statistical association with the degree of malignancy or the anatomical site (sun exposure) as summarized in Fig. 1A. No statistically significant difference in incidence was found between sun-exposed skin from patients with only benign tumors and those from patients with premalignant lesions or primary cancers (Table 1; 11 of 21 versus 10 of 18; P = 0.152, Fisher’s exact probability test). Surprisingly, 21 of 39 (54%) normal skin samples from sun-exposed sites were telomerase positive, whereas only 3 of 26 (12%) skin samples from covered sites were positive (Table 1). This difference in incidence was statistically significant (P = 0.029, Fisher’s exact probability test).

Location of Telomerase Activity in Epidermis and Hair Follicles. To localize the telomerase activity in the skin, we further examined telomerase activity both in enzymatically separated epidermis and in total skin biopsies from the same samples. Epidermal cells, separated from dermal component with trypsin, showed telomerase activity (Fig. 1B and Table 2) in 11 of 22 (50%) samples from normal skin at sun-exposed sites but in only 2 of 15 (13%) samples at covered sites. In two cases, the detected telomerase activity was localized only in the dermal part (−/+ in Table 2 and face 2 in Fig. 1B). Negativity of telomerase detection in normal epidermis is not due to inhibitors or inappropriate protein concentration.

Telomerase Activity in Hair Follicles. Most (20 of 22 volunteers) of the hair follicles of hairs plucked from the scalp showed telomerase activity (Fig. 1C). However, hair follicles plucked from some volunteers (2 of 22) did not show telomerase activity even in repeated analyses of hair follicles from several sites on the scalp and at different times (e.g., Fig. 1C, i, Lane 4). To further localize the telomerase-positive cell population in the hair follicle, we measured telomerase activity in microdissected hair follicles. Telomerase activity was localized in the regions next to the hair bulge (Fig. 1C, ii, Lane m) and beneath the hair bulge (the hair bulge region; Lane l) of plucked anagen hair follicles covered with gelatinous capsule (GC), but not at the extreme upper part of the hair follicle (Lane hs).

UV-specific CC to TT p53 Mutation. The incidence of telomerase positivity in the epidermis of skin from chronically sun-exposed sites was significantly higher than in skin from covered sites. As we have previously shown in Australian skin cancer patients (4), substantial numbers of cells already have UV-specific, CC to TT p53 gene mutations in morphologically normal skin. To examine the possible involvement of UV-specific CC to TT p53 gene mutations in morphologically normal skin for which we had already examined telomerase activity, we measured the frequency of UV-specific CC to TT
UV-ASSOCIATED TELOMERE ACTIVITY IN THE SKIN

Table 1 Telomerase activity in normal skin

<table>
<thead>
<tr>
<th>Skin sample</th>
<th>Telomerase-positive</th>
<th>Telomerase-negative</th>
<th>Incidence of positivity</th>
</tr>
</thead>
</table>
| Normal skin (n = 37)
  (n = 21) | 11                  | 10                  | 11/21 (52%)            |
| Covered (n = 16) | 2                  | 14                  | 2/16 (13%)             |
| Normal skin adjacent to premalignant tumor or cancer (n = 28)
  Sun-exposed (n = 18) | 10                  | 8                   | 10/18 (56%)           |
| Covered (n = 10) | 1                  | 9                   | 1/10 (10%)            |
| Subtotal | 21                  | 18                  | 21/39 (54%)           |
| Covered | 3                   | 23                  | 3/26 (12%)            |
| Total | 24                  | 41                  | 24/65 (37%)           |

* Face, neck, and back of hand and finger were regarded as sun-exposed body sites; other sites were regarded as covered sites.

Discussion

Activation of telomerase has been proposed to be a late event in gastric carcinogenesis (17). In colorectal cancers, telomerase activity is absent from adenomatous polyps but present in colorectal carcinoma (16). In contrast, a recent report (18) has suggested that telomerase activation is involved at an early stage in human gastrointestinal tumor development because telomerase is already activated in intestinal metaplasia and adenoma. Furthermore, telomerase activity has been detected in three of five prostatic intraepithelial type 3 neoplasias, which may be a premalignant precursor lesion of adenocarcinoma (8). In mouse skin chemical carcinogenesis, telomerase activity has been detected in papillomas (premalignant lesions of SCC) and even in hyperplastic skin before tumor formation (19). The identification of molecular events and biological consequences that precede the manifestation of epidermal malignancy may allow the early prediction and prevention of the malignant process. As described above, p53 mutations at codon 247/8 and tried to correlate it with the telomerase activity in the same samples.6 We estimated the correlation between telomerase activity and UV-specific p53 mutation in the same biopsy samples (Table 3). Among samples from sun-exposed sites, all of the samples harboring UV-specific p53 mutation (+/+ and −/− cases) were telomerase positive (7 of 7; 100%), but only 44% of telomerase-positive samples contained the UV-specific p53 mutation (+/+ and +/−; P = 0.019; χ^2 test). This bidirectional analysis suggests (although the sample size was too small) that telomerase activation may precede mutation of the p53 gene in human skin carcinogenesis.

Fig. 1. A, telomerase activity in normal skin (NS), normal skin adjacent to skin tumors, hair follicles, and various skin tumors. Lane 1, normal facial skin from a patient without skin tumors (NS sun-exposed); Lane 2, extract of skin used in Lane 1 was treated with RNase (NS + RNase); Lane 3, normal skin from covered site (NS covered); Lane 4, hair follicle (HF); Lane 5, seborrheic keratosis (SK); Lane 6, normal skin adjacent to AK of face (aNS); Lane 7, AK; Lane 8, normal skin adjacent to BD of mamma (aNS); Lane 9, BD; Lane 10, genital Paget’s disease (PD); Lane 11, BCC; Lane 12, SCC; Lane 13, metastatic lesion of SCC (mSCC); Lane 14, primary MM; Lanes 15 and 16, metastatic lesions of MM (mMM). Lanes 2, 3, 8, 13, and 15 were judged to be negative. B, telomerase activity in normal skin. Telomerase was prepared from enzymatically separated epidermis (E) from the total skin (S). C, i, telomerase activity in plucked hair follicle; ii, telomerase activity in plucked hair follicle (gelatinous capsule; GC) microdissected from upper part (hair shaft, hs), middle part (m), or lower part (l). The data in ii are representative of three independent experiments.

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we have observed telomerase activity in various premalignant lesions of skin cancers. Although the number of benign tumors we examined was limited, it appears that the telomerase activation detected may even be related to the formation of benign tumors in the skin. Similarly, BCC is usually benign because it rarely metastasizes, but most BCCs have telomerase activity. This suggests that telomerase activation is not directly related to the development of malignancy in human skin tumors. Because tumors are covered with epidermis, the detected telomerase activity in the tumors might have derived from normal epidermis rather than tumor cells. However, the facts that the positivity in tumors was irrespective of the sites and generally stronger signals than in adjacent normal skin support the idea that skin tumors have telomerase activity and that the activity is important for skin carcinogenesis. Further studies using in situ telomerase activity assay with appropriate controls will be helpful to reveal the quantitative difference of the activity in normal skin and tumors.

We found telomerase activity in a significant number of normal skin biopsy samples. Several explanations were considered: anatomically, the number of telomerase-positive cells may be higher in skin from sun-exposed sites; telomerase may be activated in situ by biological pathways associated with chronic sun exposure; or the number of cells containing telomerase may be increased by biological pathways associated with chronic sun exposure. Four explanations are possible for the activation of telomerase in normal skin and normal skin adjacent to skin lesions: (a) microscopically undetected tumor cells might remain, especially in normal skin adjacent to skin tumors. This seems unlikely, because in most cases we found a clear difference in telomerase activity between samples from sun-exposed sites and those from covered sites. Furthermore, telomerase activity was detected in normal skin from a patient with no skin tumor. However, this explanation might apply to the normal skin samples from XP patients, which had strong telomerase activity (data not shown; XP patients are extremely susceptible to UV carcinogenesis). (b) It has been shown that blood contains telomerase-positive cells (10, 11), and most of the skin tumors studied had a slight to moderate lymphoid cell infiltrate around the tumor. Therefore, the telomerase activity in both skin tumors and normal skin adjacent to skin tumors might be a result of such lymphocytic cell infiltration. However, the telomerase activity in the skin tumors seems to be unrelated to such lymphoid cell infiltration. For example, the tumors negative for telomerase also had a similar infiltrate, and there was scant infiltrate around adjacent skin that was positive for telomerase. Moreover, when infiltrating cells were counted in the histopathological samples, no correlation with telomerase activity was detected (data not shown). (c) Telomerase could be directly activated by chronic sun exposure, perhaps via mitotic stimulation of telomerase-positive cells. In the case of normal T cells, the activation of telomerase was associated with telomere RNA component expression (12). However, the molecular mechanisms by which sun exposure activates telomerase activity in these samples (Table 3, Fig. 2) are not yet well understood. (d) Chronically UV-irradiated skin, such as that of the face, has a minor population of immortal cells (telomerase-positive cells), which, although not evident on the pathological observation, grow clonally, as Greaves (20) has hypothesized. This hypothesis prompted us to examine telomerase activity in epidermis and hair follicles, where the epidermal stem cells (21, 22), which are thought to have a permanent potency for division, reside. Because most of the telomerase positivity in normal skin at sun-exposed sites is present in the epidermis and almost all of the hair follicles studied showed strong telomerase activity, we considered telomerase to be associated with such cells (stem cells). The location of hair follicle stem cells is still controversial. Cotsarelis et al. (21, 23) suggested that they may reside at the hair bulge, be quite resistant to physical removal, and tend to remain behind after hair plucking; on the other hand, Rochat et al. (24) found them to be localized beneath the hair bulge.

Our overall data consistently suggest that either activation of telomerase or clonal expansion of telomerase-positive cells by chronic sun exposure is an early feature of skin carcinogenesis, which may precede the acquisition of UV-specific p53 mutations, perhaps together with some other host factors (25, 26). This provides an attractive model for UV-associated human skin carcinogenesis (Fig. 2). In this model, (a) initially, chronic sun exposure causes the telomerase-positive cells (Tel+: perhaps stem cell) to start to proliferate; (b) these proliferating Tel+ cells acquire UV-specific p53 mutations (p53M) by chronic sun exposure (proliferation of Tel+ cells is the necessary

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**Table 3 Association of telomerase activation and/or UV-specific p53 mutation with sun exposure**

<table>
<thead>
<tr>
<th>Telomerase activity/ p53 mutation</th>
<th>Sun-exposed sites (n = 24)</th>
<th>Covered sites (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>7 (29%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>+/-</td>
<td>9 (38%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>-/+</td>
<td>0 (0%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>-/-</td>
<td>8 (33%)</td>
<td>7 (70%)</td>
</tr>
</tbody>
</table>

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**Fig. 2. Pathway suggesting telomerase activation as the first step in UV-associated human skin carcinogenesis.**
preceding step to achieve the p53 mutation); (c) the Tel"/p53M cells may be expanded clonally in the skin; and (d) finally, the Tel"/p53M cells manifest phenotypically as a precancerous lesion (Tel"/p53M).

We conclude that the activation of telomerase in the skin is associated with chronic sun exposure and occurs at an early stage of carcinogenesis, although the molecular mechanism of telomerase activation or clonal expansion of telomerase positive cells in chronically sun-exposed skin remains to be elucidated.

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