Telomerase Activity in Kaposi's Sarcoma, Squamous Cell Carcinoma, and Basal Cell Carcinoma

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Patients with acquired immune deficiency syndrome (AIDS) often develop Kaposi's sarcoma (KS), an unusual skin tumor. The malignant nature of KS has long been disputed. Telomerase activity that maintains telomere length and ensures chromosomal stability, a frequently appearing marker in human malignancies, has been proposed to play a critical role in supporting continued cell growth, hence formation of tumors. We examined telomerase activity in tissue extracts from 22 KS, 10 squamous cell carcinoma (SCC), and 22 basal cell carcinoma (BCC) using the telomeric repeat amplification protocol (TRAP). All of the tumor tissues were previously cryopreserved at -80°C. In this study, all tumor samples tested were positive for telomerase activity. Consistent with the presence of the enzyme activity, the skin tumors had relatively long telomeres. Inhibitors in the tissue extracts of some samples needed to be diluted or extracted by phenol before the enzyme activity was detected in the TRAP assay. All KS as well as two other skin carcinoma samples revealed positive telomerase activity. Our finding supports telomerase's role in tumor cell immortality and suggests the true neoplastic nature of KS.

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Telomeres, specialized structures found at the ends of eukaryotic chromosomes, appear to have important functions in chromosome protection, positioning, and replication (1, 2). Consisting of hundreds of copies of a repeat nucleotide sequence 5′-TTAGGG-3′ in vertebrates (3), telomeres may prevent the activation of DNA-damage check points and may counter the loss of terminal DNA segments that occurs during DNA replication (1, 4, 5). With each cycle of DNA replication, one end of the linear chromosome produces a short 8- to 12-bp gap generated by the removal of the RNA primer. Due to the inability of conventional DNA polymerase to fully replicate the gap just created, each chromosome end will shorten by 50 to 200 nucleotides with each cell division (6). Such shortening of telomeres provides a means of counting cell division and acts as a biological clock that associates decreasing telomere length with cell aging and senescence (7).

Telomerase, a ribonucleoprotein, adds telomeric repeats to the chromosome ends to compensate for the loss that occurs with each cell division. Maintenance of a constant telomere length ensures chromosome stability, prevents cell aging, and may confer cellular immortality. Studies of human tumor and human tumor cell lines indicate that telomerase activity may play a critical role in tumor cell growth by sustaining cellular immortality (8). In human cancer, telomerase activity was first demonstrated in ovarian carcinoma tissue (9) and has subsequently been detected in most samples from a wide range of primary human malignancies. In one study (10), cell extracts from 90 out of 101 distinct tumors representing 12 cancer types and from 98 out of 100 independent immortalized cell lines were positive for telomerase activity. In comparison, no enzyme activity could be detected in benign tumors, somatic tissues, and proliferating mortal cell lines. Because of the high prevalence in a broad range of human malignancies, telomerase activity could serve as a potential malignant marker in human tumors. Although a broad range of malignancies was investigated, telomerase activity has not been reported in Kaposi's sarcoma (KS).

Pathogenesis of KS remains uncertain. For decades, the varied histopathology and unusual presentation of KS have

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engendered debate over its neoplastic and histogenic nature. Several characteristics of KS appear to set it apart from typical neoplastic processes. Cytogenetic abnormalities are rarely observed in KS (11, 12), and spontaneous regression of a tumor may occur in a setting where lesions at other sites are progressing (13). Vascular proliferation is a prominent feature of KS, but it is also seen early in its precursor lesion (14). These observations led many to hypothesize that KS may more accurately reflect a reactive process with prominent, yet reversible, vascular hyperplasia rather than a true neoplastic process (15). KS is the most commonly occurring tumor in patients with acquired immune deficiency syndrome (AIDS) or with human immunodeficiency virus (HIV) infection. To better assess the malignant nature of this unusual tumor, we sought to determine if telomerase activity occurred frequently in KS. In this study we also examined telomerase activity in other skin cancers, particularly squamous cell carcinoma (SCC) and basal cell carcinoma (BCC).

Materials and Methods

Tumor Specimens and Patients. The fresh tissue biopsies of 54 skin tumors (22 KS, 10 SCC, and 22 BCC) obtained from clinics were stored immediately at −80°C, and some were snap-frozen in optimal cutting temperature (OCT) embedding compound and then transferred to a −80°C freezer. KS biopsies were from male patients ages 29 to 54 with HIV infection and CD4 counts less than 200 for at least 1 year. All biopsy specimens were obtained from either plaque or nodular KS lesions from the lower extremities. All but two of the patients were on prophylaxis for opportunistic infections. However, none of the patients was on nucleoside analogue reverse transcriptase (RT) inhibitors, non-nucleoside reverse transcriptase (NRT) inhibitors, or HIV-1 proteinase inhibitors (PI), although some had previously been on RT. All tumors of SCC and BCC were from chronically sun-exposed skin with moderate to severe solar elastosis histopathologically. Tumors of SCC were either well-differentiated or moderately well-differentiated tumors with no evidence of metastasis spread from the head and neck and distal upper extremities. The tumors of BCC were all nodular BCC biopsied from the head, neck, and trunk.

Cells and Cell Cultures. Cells from the primary culture of human skin fibroblasts and the suspension culture of Epstein-Barr virus (EBV)-immortalized human B lymphocytes (clone 1656) were used as negative controls for the telomerase TRAP assay in this study. Transformed human embryonal kidney cell line 293 (ATCC CRL 1573, American Type Culture Collection, Rockville, MD) and human cervical squamous carcinoma cell line SiHa (ATCC HTB 35) were used as the positive controls. All cells were grown in cultures using RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco-BRL, Gaithersburg, MD) and were kept in a 5% CO_2 incubator at 37°C. Cells were harvested from the logarithmically growing cultures, pelleted by centrifugation at 2000 rpm for 20 min, and kept in −80°C freezer.

Extract Preparation. Individual tissues were washed once with 0.5 ml of cold lysis extraction buffer [10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, and 0.5% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS)] on ice to remove traces of blood and embedding compound. Each tissue (50–150 mg) was minced with sterile surgical scissors and then homogenized using motor-driven disposable pestles in 200 μl of lysis extraction buffer on ice. The samples were incubated on ice for 30 min and were centrifuged at 13,000*g* for 30 min at 4°C. The supernatants were collected and immediately stored at −80°C. Tissue culture cell extracts were similarly prepared using 10^8 cell pellets. The protein concentrations of the extracts were between 0.5 to 1.6 μg/μl.

Telomerase Assay. Telomerase activity was determined using the TRAP assay developed by others (10) with some modification. Telomerase-mediated extension of the TS (5′-AATCCGTCGAGCAGAGTT-3′) primer was carried out in a 50-μl reaction mixture containing 1 or 2 μl of cell extract, 1× reaction buffer (20 mM Tris-HCl, pH 8.3, 68 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and 0.005% Tween 20), 0.1 μg of TS primer, 1 μg of T4 gene 32 protein, 50 μM of each deoxynucleotide triphosphate, and 2 units of Taq DNA polymerase at 23°C for 60 min. After extension, 2 μl of solution containing 0.1 μg of CX [5′-$(CCCTTA)_3CCCTAA$-3′] primer and 0.4 μl of α-^{32}P-dCTP (10 μCi/μl, 3000 Ci/mmol) was added to the reaction and subsequent PCR was carried out with 30 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 90 sec. One-half of the volume of the PCR reaction was analyzed on 10% nondenaturing polyacrylamide gels in 0.5× Tris-borate-EDTA buffer at 150 V for 2 hr. Then gels were dried and exposed to film.

Analysis of Terminal Restrictive Fragments. Genomic DNAs were prepared from frozen tumor tissues. Briefly, 100 to 200 mg of tissue was minced and homogenized in 1 ml of lysis buffer (10 mM Tris, pH 8.0, 0.15 M NaCl, 25 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulfate, and 0.2 mg/ml of proteinase K) and was then incubated overnight at 55°C. Samples were extracted once with phenol and once with phenol:chloroform:isoamyl alcohol (25:24:1), and then precipitated with ethanol. DNA pellets were resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) and digested with MseI plus MnlI to release telomere fragments. Approximately 1 μg of digested DNA per lane was loaded onto an 0.8% agarose gel. Separated DNA was electrically transferred to a nylon membrane and the membrane was hybridized with the ^{32}P-labeled oligonucleotide $(TTAGGG)_3$. Telomere length was estimated from migration of DNA size markers run parallel with the samples. Mean telomere lengths were determined by analysis of autoradiographs. Briefly, the autoradiographs were

scanned and the peak optical density reading was calculated with UN-SCAN-IT (Silk Scientific Corporation, Orem, UT)

Results

We included immortalized tissue culture cells (transformed human embryonal kidney cell line 293 and human cervical SCC line SiHa) as positive controls, and primary culture cells (human skin fibroblasts and EBV-immortalized human B lymphocytes) were used as negative controls for telomerase activity in all of our assays. In this study, initially 9 out of 22 KS, 14 out of 22 BCC, and 5 out of 10 SCC tissue extracts tested positive for telomerase activity. Figure 1 shows the characteristic ladder pattern of positive telomerase activity in KS, BCC, and SCC tissue extracts in the TRAP assay. Inhibitors that could interfere with the TRAP assay are often present in tissue extracts (16). To minimize the potential inhibitory effect in the tissue extracts, we re-examined the enzyme activity after the tissue extracts were further diluted to lower the concentration of potential inhibitor(s) in the preparations. A simple dilution of the tissue extract preparations allowed telomerase activity to be detected in an additional 10 KS, 6 BCC, and 3 SCC samples (Table I).

To remove potential inhibitors from the preparations of tissue extracts, we extracted the reaction mixtures once with phenol and once with phenol/chloroform, and then precipitated them with alcohol after telomerase-mediated extension (17). The pellets were then suspended to continue PCR in the TRAP assay. After additional phenol/chloroform treatment, the remaining tissue samples of 3 KS, 2 BCC, and 2 SCC also tested positive for the enzyme activity. Thus, all 54 tissue samples from 22 KS, 22 BCC, and 10 SCC were positive for the telomerase activity (Table I).

For skin tumors with sufficient tissue for further study (6 KS, 7 BCC, and 6 SCC), genomic DNAs were prepared for the telomere length examination. The terminal restrictive fragments (TRF) of the telomere DNA were revealed by hybridization with a probe of telomere-specific sequence. The representative result is shown in Figure 2. The TRF ranged between 5 and 15 KB. The mean TRF lengths for KS, BCC, and SCC were determined to be 12.1, 12.6, and 10.4 kb, respectively. No significant difference existed between KS, BCC, and SCC.

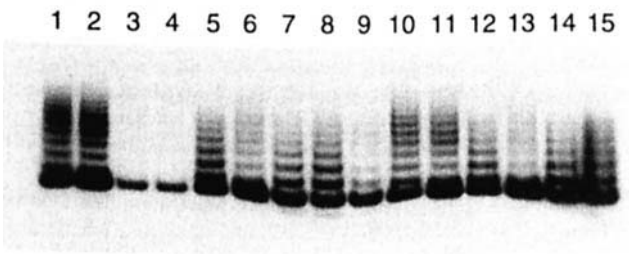

Figure 1. Representative TRAP assay for telomerase activity in extracts of immortalized cell lines (293 and SiHa cells), primary human cell cultures (lymphocytes and skin fibroblasts), and tumors. Positive controls: lane 1, 293 cells; lane 2, SiHa human cervical carcinoma cells; negative controls: lane 3, human lymphocytes, lane 4, human skin fibroblasts; BCC: lanes 5 and 6; SCC: lanes 7 and 8; KS: lanes 9 through 15.

Table I. Telomerase Positive Tissue Extracts after Dilution or Phenol Extraction

	No dilution	5- to 10-fold dilution	Phenol extraction	Total
KS	9	10	3	22
SCC	5	3	2	10
BCC	14	6	2	22

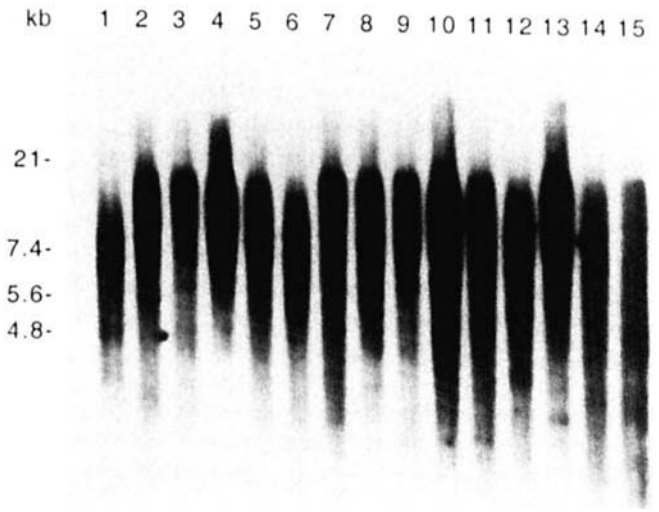

Figure 2. Length of terminal restrict fragments of representative KS samples (lanes 1–5), BCC (lanes 6–10), and SCC (lanes 11–15).

Discussion

It is of vast biomedical interest that a high proportion (about 80%–90%) of tumors in animals and humans have been found to contain telomerase activity (18, 19). Although the mechanisms of activation of telomerase in tumors are still not fully understood, many scientists believe that activation of the enzyme is required for uncontrolled continuous cell proliferation, the hallmark of tumors. Thus, positive telomerase activity could be an important tumor marker. A new cancer paradigm is raised based on telomerase activation and oncogenesis. Some scientists have also proposed a new approach to treating a wide range of tumors, using an anti-telomerase strategy (19, 20). In the present study, our finding that 100% of 22 KS, 10 SCC, and 22 BCC samples tested contain telomerase activity further supports the possible role of telomerase in tumor development.

As previously described by other laboratories, inhibitors that interfere with the assay for telomerase activity are often present in tissue extracts. Fortunately, these inhibitory effects either can be minimized by a serial dilution or can be removed by applying an additional step of phenol/chloroform extraction. After dilution or successful removal of tissue inhibitor(s), all 22 BCC and 10 SCC were positive for the telomerase enzyme activity. The length of telomeres of skin tumors was also found to be rather long (12.1, 12.6,

and 10.4 kb for KS, BCC, and SCC, respectively). However, many studies have indicated that there is apparently no significant relationship between telomerase status and the size of telomere lengths (21–23).

Our finding is consistent with a recent report that found telomerase activity in high frequency in BCC (24). However, Parris *et al.* (21) reported a lower prevalence of telomerase activity in SCC. They claimed that large parts of telomerase-negative SCC did not result from inactivation of protein samples and inhibitors. It is not clear why they detected telomerase activity in only 25% of SCC. Our study and others (24, 25) found a high frequency of positive telomerase in SCC. It was somewhat surprising to find that all 22 KS tumors tested also had telomerase activity. The fact that none of the biopsied patients were on RT inhibitors may have some significance, since these drugs can inhibit the enzymatic component of the telomerase (26). The widespread use of RT inhibitors even prior to institution of PIs may, in part, explain the decreasing incidence of KS that occurred earlier in the HIV- 1 epidemic, more so than the use of PIs (27). The addition of PI, with its improvement in the immunologic status of the patient, appears to have further decreased the incidence of KS (28).

In this context, it is important to note that the telomerase model in oncogenesis has become more complex. Many recent studies have shown that detection of the enzyme activity could simply be correlated to the growth index of cells. For example, low levels of enzyme activity in mouse mammary tissue and skin samples became greatly elevated when the cells were grown in short-term culture (29). Normal skin samples have very low levels of activity. On the other hand, when skin layers are dissected, a high level of telomerase activity is detected in the proliferating basal cells, whereas the quiescent dermis is telomerase negative (30). Thus, this enzyme activity is likely to be growth regulated in various human tissues *in vivo*. However, two telomerase-negative normal human cell types, retinal pigment epithelial cells and foreskin fibroblasts, were recently transfected with vector encoding the telomerase catalytic subunit (31). In contrast to telomerase-negative control clones that showed telomere shortening and senescence, telomerase-expressing clones had elongated telomeres and they divided vigorously. These telomerase-expressing clones had a normal karyotype and had already exceeded their normal life span by at least 20 doublings (31). The result has established a causal relationship between positive telomerase activity and prolonged cellular senescence. More recent studies have further implicated telomerase's role in cell immortalization, but not necessarily in malignant transformation (32, 33).

Our finding of relatively long telomeres and prominent telomerase activity at high frequency in KS would indicate that KS cells are immortalized, not the result of reactive hyperplasia of various endothelial or mesenchymal cells. Tumors of BCC and SCC are evidently also immortalized with upregulated telomerase and long telomeres. Upregulation of telomerase in chronically sun-exposed skin appears to be an early event. It occurs even prior to UVB-induced signature P53 mutation (25, 34). Thus, upregulation of telomerase may be found early at high frequency in UVB-induced tumors such as BCC and SCC.

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