Endocrine, *vol. 9, no. 2, 133–138, October 1998* 0969–711X/98/9:133–138/$9.50

Review

Telomeres and Telomerase in Endocrine Pathology

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Telomeres representing repetitive DNA sequences of chromosome ends are necessary for maintaining chromosomal integrity. The enzyme telomerase synthesizes *de novo* telomeric repeats and incorporates them onto the DNA 3'-ends of chromosomes. Stability of chromosome ends and activation of telomerase are elementary requirements for cell immortalization and tumor progression. The telomeric length and telomerase activity have been recently studied in several human neoplasms, including those of endocrine tissues. Assessment of telomerase activity may help to distinguish normal or hyperplastic from neoplastic tissues. Inhibition or inactivation of telomerase activity may provide novel strategies for cancer therapy.

Key Words: Cancer; endocrine glands; immortality; telomerase; telomeres; tumor.

Introduction

During the last decade, evidence has emerged that telomeric repeats of chromosome ends and telomerase activity may be different in benign and malignant tumors. The presence of highly expressed telomerase activity in the substantial majority of human cancers has created great interest in oncology. Currently, telomeres and telomerase represent objectives of a hot area of research; they may be useful novel markers in distinguishing malignant from normal and hyperplastic lesions. However, owing to technical limitations of the available methods, until recently, the interpretation of telomeres and telomerase activity was not possible in archival material. This article reviews recent information on telomere and telomerase in endocrine tissues.

Received July 17, 1998; Accepted July 24, 1998.

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Telomeres and Telomeric Length

Early genetic studies have shown that chromosome ends or telomeres are necessary for maintaining chromosomal integrity *(1,2)*. Recent studies highlight their properties at the molecular level. Telomeres in most species represent complex protein–DNA structures, which cap the ends of chromosomes. Together with secondary structures and specific associated proteins, they stabilize the linear chromosomal DNA molecule. In addition, telomeres facilitate chromosome attachment to the nuclear membrane. Human telomeres consist of tandem arrays of short hexameric sequence $(3'\text{-TTAGGG-}5')_n$ repeats. They are rich in G bases, and their length ranges between 100 and 300 kb *(3)*. The telomeric sequences are double-stranded, except for the extreme 3'-end, where a short-single stranded stretch is formed.

Telomeres are noncode sequences, and therefore, they were initially thought to have little functional significance. However, replication of telomeres and regulation of telomeric length have recently become an important area in oncology. Telomeres are essential for complete replication of eukaryotic chromosomes. The telomere length is stable in germ cells. In contrast, chromosomes of somatic cells have a definite proliferative capacity and lose a small amount of telomeric sequences that accompany DNA replication with each successive cell division. This loss is attributed to failure of mechanisms accounting for the replication of the end sequences of the lagging DNA strand. Somatic cells gradually lose their telomeric repeats, and when their length reaches a certain critical level, a DNA damage response pathway is activated. By inducing cell-cycle arrest, the cells stop dividing *(4)*. Cell division seems to control life-span. The regular telomere loss may serve as a mitotic clock in the cellular senescence program by eventually counting the signal cell-cycling exit *(5)*. Telomere damage by cell division through "end replication" problems may lead to telomeric-associated cytogenetic aberrations *(6)*. In addition, telomeric deletions resulting in genetic instability may be important in carcinogenesis. Therefore, telomeres are essential in providing important protection

from illegitimate recombination, and they master gene loss of the subtelomeric regions *(7)*.

Telomerase and Telomerase Activity

The ribonucleoprotein enzyme telomerase, a complex protein with an RNA component, is a reverse transcriptase, which synthesizes *de novo* telomeric repeats and adds them onto the DNA 3'-ends of chromosomes *(8)*. Human somatic cells do not normally possess telomerase activity *(9)*. In contrast, human germ cells maintain a stable telomere length through the action of telomerase. The same is true for "immortal" cells, such as tumor cell lines, and established human and rodent cells. In these cells, the activation of telomerase inhibits telomere decline by extending the telomeric ends *(10)*. However, the stability of telomeric length suggests the existence of a regulatory mechanism for limiting telomere elongation by telomerase. In the absence of telomerase, telomeres gradually shorten with each cell division and reach the critical point associated with cellular senescene, also known as Hayflink limit *(11)*. It has been found that suppression of telomerase activity leads to instabilization of chromosomes ends, resulting in cell growth arrest and subsequent death. Stabilization of telomere length appears to be necessary for cell immortalization. Thus, stability of telomerase activity is characteristic of cell proliferation and immortality *(10)*. It has been found that immortal cells repress telomerase activity when they become quiescent or differentiate to a postmitotic state *(12)*.

Evidence of telomerase reactivation has been recently demonstrated in approx 90% of various types of primary human cancers *(5)*. Telomerase activity is thought to play a significant role in the proliferative ability of cells, and it seems to be necessary for tumor progression. However, upregulation of telomerase activity is required to maintain telomere length in cancer cells. Mutations occurring in the telomerase repression pathway lead to upregulation or to reactivation of telomerase. Therefore, telomerase is considered to be a valuable novel marker of human neoplasms.

Demonstration Techniques for Telomeres and Telomerase

Telomeres can be visualized by fluorescence *in situ* hybridization (FISH) technique, particularly in metaphase spreads *(13,14)*. Rearrangements involving telomeres can be detected by various polymerase chain reaction (PCR) protocols and can be visualized by FISH technique. An initial study exploits telomere-specific probes and permits simultaneous analysis on as single microscopic slide of all chromosome subtelomeric regions for mutation events *(15)*. Primed *in situ* (PRINS) labeling represents an alternative to standard FISH technique and can be used for the detection of centromeric chromosome sequences *in situ (16)*.

An *in situ* PCR assay has been recently introduced to detect telomerase activity at the cellular level *(17)*. The technique utilizes amplification of telomeric repeats using fluorescein-labeled primers and subsequent study under fluorescence microscope. Although this technique gave excellent results in peripheral blood mononuclear cells and spreads from cell lines, it was unsuccessful on frozen sections of fresh material. It seems that additional modifications are needed to prevent solubilization/diffusion of the enzyme and to increase permeability of the samples.

Southern blot analysis is used to estimate telomere restriction fragment (TRF) length *(18)*. The fragments of extracted genomic DNA are subjected to gel electrophoresis and labeled with radioactive $(TTAGGG)_n$ telomeric probe. The length of the restricted fragments is estimated from the maximal signal.

For detection of telomerase activity, the telomeric repeat amplification protocol (TRAP) was initially introduced in 1985 *(19)*. The principle of this assay is based on the ability of telomeres to act as template and to incorporate telomeric repeats onto the 3'-end of a specifically designed primer. The protocol was later improved by increasing up to 10^4-fold its sensitivity of the detection level using ^{32}P-labeling and autoradiographic detection of the PCR-amplified product *(9)*. The TRAP assay still remains the most widely used to demonstrate telomerase activity, and it can be applied on fresh and frozen cells, and tissue extracts as well. However, the time required to complete the assay, the poor resolution of the results, and the hazards of radioactive material represent disadvantages of this method, which can be alleviated by the modified fluorescent TRAP (F-TRAP) assay *(20)*. This newly modified method, which employs fluorescein instead of radioisotopic labeling, is time-effective, safe, and offers higher resolution and increased sensitivity than the standard TRAP assay. However, the presence of lymphocytes, which have telomerase activity, requires cautious interpretation in tissue extracts.

In a preliminary series of experiments, the *in situ* hybridization of telomerase RNA was successfully tested on paraffin section. The results may encourage researchers to improve and apply the technique in archival material *(21)*.

Overview of Endocrine Studies

Pituitary

In a single study, among 87 intracranial tumors, 22 pituitary adenomas were recently investigated for telomerase activity. They included eight somatotroph, three lactotroph, eight gonadotroph, two null cell, and one silent corticotroph adenoma. All invasive and noninvasive tumors were found to be negative for telomerase expression *(22)*.

Thyroid

Follicular thyroid adenomas are difficult to separate from carcinomas when the tumors are uninodular on the basis of capsular or vascular invasion and impossible in fine-needle aspiration (FNA) biopsies. In a recent report,

the TRAP assay was applied on frozen tissue of 44 follicular tumors. Telomerase activity was found in all 11 carcinomas (100%), 5 of 23 benign follicular adenomas, and 3 of 10 hyperplastic nodules, whereas all 22 normal thyroid tissue samples were negative for teleomerase activity *(23)*. In the same report, a pilot study on FNA biopsies suspicious for thyroid cancer revealed telomerase activity in four papillary carcinomas and negative in two benign adenomas, which were subsequently diagnosed by histology. In a similar study, telomerase activity was detected in 11 of 20 thyroid carcinomas, including 10 of 14 papillary carcinomas and 1 Hürthle cell carcinoma *(24)*. The activity was correlated with tumor invasiveness (vascular and/or capsular) in six of seven invasive papillary carcinomas, but not in benign adenomas. However, in both studies, false-positive results were obtained in extranodular tissues containing lymphocytic infiltrates, which are known to have telomerase activity. In another similar study, telomerase activity was detected in 3 of 15 (20%) papillary carcinomas, in 4 of 6 (66.5%) follicular carcinomas, and in 2 of 3 (66.5%) undifferentiated carcinomas *(25)*. Only 1 of 12 adenomas showed telomerase expression. Telomerase activity may thus be valuable diagnostic marker for malignant and invasive thyroid tumors, and it may play a role in predicting tumors of more aggressive behavior, such as follicular and undifferentiated carcinomas.

Adrenals

Almost all 100 cases of adrenal neuroblastomas (94%) expressed telomerase activity *(26)*. In contrast, telomerase activity was not detected in normal adrenal tissues or benign ganglioneuromas, suggesting an important role for telomerase in neuroblastoma development. Neuroblastomas with high telomerase activity exhibited additional genetic changes, such as *N-myc* amplification, and an unfavorable prognosis, whereas tumors with low telomerase activity showed no such genetic changes and were associated with a favorable prognosis. Tumor regression was noted in three neuroblastomas lacking telomerase activity. It seems that telomerase expression may be required as a critical step in the multigenetic process of pathogenesis of neuroblastomas.

Pancreas

In a single study based on human material, telomerase activity was detected in 41 (95%) of 43 pancreatic cancers, but in none of 11 benign pancreatic tumors, whereas only 1 of 3 pancreatitis samples was positive *(27)*. Low levels of telomerase activity were detected in 5 (14%) of 36 adjacent "normal" pancreatic tissues, probably owing to occult microinvasion. Telomerase activity was examined in 12 brushing samples of the pancreatic duct, and all 8 with pancreatic cancer showed detectable telomerase activity, whereas the 4 benign lesions (cystadenoma and pancreatitis) were negative. These findings suggest that telomerase activity may be useful in the diagnosis of pancreatic cancer.

Gonads

The temporal regulation of expression of telomerase activity was examined in developmental studies of human germline and somatic tissues *(28)*. Telomerase activity was detected in fetal, newborn, and adult testes and ovaries, but not in mature spermatozoa or oocytes. Blastocysts expressed high levels of telomerase activity as compared with most human somatic tissues at 16–20 wk of development with the exception of human brain tissue. This activity could no longer be detected in somatic tissues examined after the neonatal period. Explants of fetal tissues in primary cell cultures showed a dramatic decline in telomerase activity, which became undetectable after the first passage in vitro. Elucidation of the pathways involved in the regulation and repression of telomerase activity during development may provide valuable information in the better understanding of the consequences of cellular aging and cancer cell survival.

The cytogenetic evolution of multiple cell lines involving clonal telomeric associations of the Y chromosome has been described in the gonadal tissue of a 10-yr-old girl with mosaic Ullrich-Turner syndrome. G-band analysis of all tissues showed at least two separate cell lines. Analysis of left gonadal tissue showed the evolution of two additional cell line. FISH analysis of interphase nuclei from uncultured gonadal tissue confirmed the aneuploidy in both gonads. This case provides evidence that clonal telomeric fusions may be associated with chromosome malsegregation and with subsequent evolution of unstable karyotypes *(29)*.

Testes

Since human telomeric repeats and telomerase RNA component are guanosine-rich, the possibility of whether the sequence-specific, G-Pt-G, crosslinking agent cisplatin is capable of inhibiting telomerase activity was examined. Telomerase activity was measured by the TRAP assay in cisplatin-treated testicular cancer cell extracts and RT-PCR was used to examine telomerase RNA component expression *(30)*. Cisplatin was found to reduce telomerase activity in a specific and concentration-dependent manner in human testicular tumor cells, whereas doxorubicin, bleomycin, methotrexate, melphalan, and transplatin were not effective. It is conceivable that cisplatin used in the treatment of testicular cancer may cause inhibition of telomerase.

Ovaries

Cytogenetic analysis of seven established cell lines from human ovarian surface epithelium revealed a high frequency of telomeric associations ranging from 30 to 100% of the metaphases examined, with a higher rate of the short arms of chromosomes 16, 19, 21, and 22. Telomeric association with other chromosomal ends appeared to be random *(31)*.

Telomerase activity and telomere length in metastatic cells of ovarian carcinoma were measured to assess the relevance of enzyme activity and telomere stability in tumor

formation and progression *(32)*. The authors reported that extremely short telomeres were maintained in these cells, and that telomerase was expressed by the tumor cells, but not by isogenic nontumorous cells. Telomerase activity was also detected in 88% of 16 ovarian malignancies examined, but not in normal tissues or in benign proliferative gynecologic lesions *(33)*. In a study of 41 women with ovarian tumors, telomerase activity was detected in 23 of 25 malignant tumors (92%), in 1 of 6 borderline malignant tumors (16.7%), and in 2 of 10 benign tumors (20%) *(34)*. Terminal restriction fragment length ranged between 8 and 13 kbp for normal ovaries and was correlated with the stage in most tumors. Thus, it appears that telomerase activity may be a useful marker in the diagnosis of ovarian tumors. These findings suggest that tumor progression is ultimately dependent on activation of telomerase and that telomerase inhibitors may be effective antitumor drugs. In another report of 64 consecutive ovarian tumors, including 20 carcinomas, 17 low malignant potential tumors, and 27 cystadenomas, were studied to verify whether the expression of telomerase can distinguish ovarian cystadenomas from carcinomas *(35)*. Telomerase activity was detected in all carcinomas and low malignant potential tumors. In contrast, telomerase was undetectable in 19 of the 24 cystadenomas. All five papillary cystadenomas were telomerase-positive. Telomerase expression in ovarian low malignant potential tumors, indicates the need to separate these tumors from cystadenomas. Telomerase expression in papillary cystadenomas suggests that such tumors may be reclassified as variants of the low malignant potential tumor category.

Prostate

All five cell lines of human prostate cancer examined for telomerase activity (DU145, LNCaP, PC-3, PPC1, and TSU) showed very high telomerase activity *(36,37)*. Further studies evaluating telomere length in two of these cell lines (LNCaP and DU145) showed a specific telomere length for each cell line *(37)*.

Strong telomerase activity associated with human prostate cancer was reported in 21 of the 25 (84%) adenocarcinomas from radical prostatectomies *(36)*. Four lymph nodes with metastases were strongly positive for telomerase activity. Only 3 of the 25 tissues (12%) obtained from benign prostatic hyperplasia adjacent to cancerous prostates were weakly positive. No telomerase activity was detected in 10 samples from patients who underwent surgery solely for benign prostatic hyperplasia. Telomeric lengths measured in 27 samples of cancer tissue from 9 radical prostatectomies were significantly and consistently shorter than either the adjacent normal or adjacent benign hyperplastic samples of the same glands. In another study, telomerase activity was examined by PCR-based TRAP assay in prostate tissues *(38)*. From 31 primary prostate cancers, 28 (90%) displayed telomerase activity. Poorly differentiated prostate cancer showed more frequently high levels of telomerase activity. Samples taken from benign prostatic hyperplasias or normal prostates were negative for telomerase. One of 10 samples (10%) obtained from nontumorous prostate adjacent to cancer tissue showed weak positivity. Extremely high telomerase activity was noted in four lymph nodes and in one bone metastasis. In similar study, telomerase activity was detected in 8 of 9 (89%) prostate cancer biopsies and in 6 of 16 benign prostatic hyperplasia biopsies, but not in 11 apparently normal prostates *(39)*. The presence of telomerase in a subset of benign prostatic hyperplasia may indicate early cancer development and suggests that enzyme activity may be of use as a biomarker in this particular group of patients *(37)*. These results indicate that telomerase activity, and telomere lengths, represent potential diagnostic markers for prostate cancer.

Breast

During the in vitro establishment of two new breast cancer cell lines from pleural effusions, both showed a marked decrease of the upper border range of telomeric repeat length distribution, whereas the lower border remained within a constant range *(40)*. However, decrease of repeat length was not accompanied by an increased incidence of telomeric associations or fusions, indicating that a constant telomeric repeat length does not necessarily constitute a characteristic feature of immortalized cells.

Telomerase activity and TRF length were determined in breast cancers. Several studies were reported with slightly different results, and no correlation was found with most of the known prognostic factors. A widely varying mean TRF length was found in 85 human breast cancer samples, reflecting a unimodal distribution *(41)*. No significant correlation was noted between TRF length of the tumors and histologic grade, tumor volume, lymph node status, steroid receptor status, or age of patient. In another study, the clinical significance of telomeric deletions and c-*erb*B-2 gene amplification was investigated in patients with breast disorders *(42)*. Significant reductions in telomeric length and concentration were observed in all breast lesions with no significant differences among breast carcinomas, fibroadenomas, or gynecomastia. Significant correlation was found between the degree of telomeric deletion and histologic grade, mostly in Grade 3 carcinomas. The degree of telomeric deletion did not correlate with c-*erb*B-2 gene amplification, tumor size, clinical stage, steroid receptors, or prognosis. Shorter telomeric length was noted in lymph node-negative compared to positive tumors. In a recent study, telomerase expression was detected in 22 of 28 (79%) primary breast cancers, including 16 of 22 (73%) carcinomas positive for axillary metastases and 6 of 6 (100%) node-negative carcinomas *(43)*. In addition, it was found in 1 of 9 (11%) fibroadenomas, but was negative in 13 normal breast tissues. However, no statistical association in telomerase expression was noted between axillary node-

negative and node-positive primary breast cancers. Again, there was no statistical difference between telomerase activity and tumor size or hormonal status. In an other study, 52 of 71 (73%) breast carcinomas and only 1 of 15 (6.5%) fibroadenomas were positive for telomerase activity, whereas all 6 samples of normal breast tissue were negative *(44)*. Invasive ductal carcinomas were more frequently positive than invasive lobular carcinomas. No correlation was noted between telomerase activity and tumor size or the occurrence of lymph node metastases. These findings indicate, but do not confirm that telomeric reduction may promote breast cancer progression. Finally, the value of telomerase activity was tested in FNA biopsies. Ten of 15 (66.5%) cytologically positive aspirates showed strong telomerase activity; 26 of 29 (90%) benign aspirates were negative, and the remaining 3 showed only borderline activity *(44)*. In three cases in which the cytologic observations were inconclusive, telomerase results helped to establish the cancer diagnosis. It seems that telomerase activity represents a sensitive assay, which might be useful in supplementing microscopic cytopathology in FNA biopsies.

Uterus

Telomerase activity detected in 60 normal endometrial biopsies was much higher and more frequent in the substantial majority of samples from the proliferative phase of the menstrual cycle (95%). Immunohistochemical assessment of cell proliferation using antibody against the proliferating cell nuclear antigen revealed correlation between telomerase activity and endometrial cell proliferation *(45)*. These findings indicate that telomerase is a regulated enzyme linked to cellular proliferation and that sex steroid hormones may mediate this regulation. In a similar study, telomerase activity detected in the normal endometrium was maximal at the late-proliferative phase to mid-secretory phase, and was absent or extremely low at early-proliferative phase and late-secretory phase. In addition, all endometrial simple hyperplasias (16 of 16) and most cancers (28 of 30) showed telomerase activity. No telomerase activity was detected in endometrium of either pregnant or postmenopausal women in the absence of hyperplasia *(46)*. These results suggest that reactivated telomerase activity in the endometrium of postmenopausal patients can be used as a novel marker for early endometrial cancer diagnosis. Another study of 34 human endometrial tissues showed similar results: 19 of 20 (95%) endometrial carcinomas, and 8 of 8 (100%) benign endometrial tissues from premenopausal women exhibited strong telomerase activity, whereas 6 of 6 (100%) benign endometrial tissues from postmenopausal women showed only weak telomerase activity. Furthermore, no correlation was found between telomerase activity and tumor grade, depth of invasion, or DNA content *(47)*.

Perspectives

Research accomplished during the last decade on telomerase activity and the regulatory mechanisms to maintain stability of telomere length in human cancer significantly contributed to a deeper understanding of the nature of immortalized and malignant cells. Assessment of telomerase activity may be used to separate malignant from benign lesions, to identify certain morphologic cancer subcategories, and to predict aggressive clinical behavior and prognosis. Further studies may result in novel, revolutionary therapeutic modalities, such as antisense therapy *(48,49)*. Telomeres may be ideal targets for cancer treatment. Telomerase inhibition has been proposed as a potentially selective target for therapeutic intervention *(30)*. Indeed, suppression or inhibition of telomerase activity in cancer patients may result in tumor remission. New anticancer treatment strategies may lead to the identification of telomerase inhibitors, which can prevent cell proliferation *(50)*.

Acknowledgments

This work was supported in part by grants E-52/96 of the National Health Council of Greece and MA-6349 of the Medical Research Council of Canada.

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