Replicative Senescence of Normal Human Oral Keratinocytes Is Associated with the Loss of Telomerase Activity without Shortening of Telomeres

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Abstract
Telomerase activity was analyzed in 7 different cultures of secondary normal human oral keratinocytes (NHOKs), 1 normal human oral epithelial tissue specimen, 1 immortalized human oral keratinocyte (HOK) cell line, and 10 human oral cancer cell lines using the PCR-based telomeric repeat amplification protocol assay. Telomerase activity was found in all tested cells and tissue, but the activity in NHOKs and epithelial tissue was lower than that in other tested cell lines. Inasmuch as continued subculture of NHOKs results in replicative senescence, we investigated the association between telomerase activity and replicative senescence by evaluating the enzyme activity in NHOK cultures with different population doubling levels. Three different NHOK cultures were independently subcultured until these cells reached the postmitotic stage. Unlike in fibroblasts derived from the human oral cavity, significant telomerase activity was detected in rapidly proliferating NHOKs, and telomerase activity was barely detectable in the keratinocytes near and at senescence. However, the terminal restriction fragment consisting of telomeric DNA was found to be constantly maintained at ~6.0 kilobases in NHOKs without any detectable shortening of telomeres by subcultures. Intracellular p53 and p21WAF1/CIP1 protein levels in NHOKs were gradually and significantly diminished by the passage of cells. These data indicate that actively proliferating NHOKs contain telomerase activity and that replicative senescence of NHOKs is associated with the loss of telomerase activity without shortening of telomeres. However, replicative senescence of NHOKs is apparently not linked to an accumulation of wild-type p53 and/or p21WAF1/CIP1 proteins in these cells.

Introduction
Eukaryotic chromosomes are flanked with hexameric repeat sequences known as telomeres (1, 2). Telomeric repeats are essential for maintaining the stability and integrity of chromosomes and tightly involved in protecting cellular DNA from exonucleases and ligases that may cause diverse chromosomal abnormalities in the absence of telomeres (3–6). The length of the telomeric repeat is an important aspect of cellular aging and senescence in that cells lose their proliferative capacity when telomeres shorten beyond critical length after several rounds of DNA replication (7–10).

The telomeric sequence is maintained at a certain length in cancer cells, whereas it progressively shortens in normal somatic cells with aging (11–15). This difference is mainly attributed to telomerase, a ribonucleoprotein complex with reverse transcriptase motifs (16, 17). Telomerase activity is found in the vast majority of established immortalized and cancer cell lines and is absent in all but a few normal somatic cells (18–24). Recently, telomerase activity was detected in normal human foreskin epidermis in a partially purified basal cell population containing stem cells, which undergo constant reproduction and differentiation (19, 23). The suprabasal population of the epithelial stratum, however, failed to demonstrate telomerase activity (23). Taken together, these observations suggest that there may be a positive association between telomerase activity and the proliferative capacity of normal human epithelial cells, because the basal cells can proliferate whereas the cells from suprabasal layers cannot (19).

The loss of cell proliferative capacity at senescence has been shown to be linked to the increased cellular level of wt4 p53 (a tumor suppressor protein) and p21WAF1/CIP1 (a universal inhibitor of cyclin-dependent kinase) at least in normal HDFs and human tumor cells in which the tumor suppressor protein is overexpressed (25–32). Inasmuch as down-regulation of telomerase activity may be associated with the loss of proliferative capacity of cells, the following has been hypothesized. The absence of telomerase activity in senescent cells might be correlated to the accumulation of wt p53 and p21WAF1/CIP1 in such a way that wt p53 directly or indirectly

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4 The abbreviations used are: wt, wild type; HDF, human diploid fibroblast; HPV, human papillomavirus; NHOK, normal human oral keratinocyte; TRF, terminal restriction fragment; TRFL, TRF length; PDL, population doubling level; HOK, human oral keratinocyte; BaP, benzo[a]pyrene; BaP-T, BaP treatment of tumorigenic counterpart; CHAPS, 3-[3-cholamidopropyl]dimethylammonium)-1-propanesulfonate; TRAP, telomeric repeat amplification protocol; NHOF, normal human gingival fibroblast; PCNA, proliferating cell nuclear antigen; FBS, fetal bovine serum.
represses telomerase activity during cell aging. This hypothesis has been examined and refuted by experiment in which the mutant E6 protein of type 16 HPV, which cannot bind and promote the degradation of wt p53, still prevents the loss of telomerase activity in human epithelial cells (33). Previously, a rather indirect relationship between telomerase and p53/p21WAF1/CIP1 has been formulated in that the lack of telomerase activity results in the induction of p53 and/or p21WAF1/CIP1 at cellular senescence due to the progressive shortening of telomere DNA during the replicative life span of normal somatic cells.

Vaziri and Benchimol (25) proposed a model that explains the role of telomere shortening in replicative senescence of HDFs. According to this model, shortening of telomeres beyond the critical length induces DNA damage, which may result in the elevation of cellular p53 and/or p21WAF1/CIP1 levels, causing cell cycle arrest and senescence. However, other studies report no alteration in the level of p53 during replicative senescence of fibroblasts (34, 35). Rerevaluation of this model is thus necessary to understand the mechanism of replicative senescence of normal human epithelial cells that, unlike fibroblasts, possess functional telomerase. To address this issue, we first determined the presence of telomerase activity in NHOKs and various human oral cancer and immortalized cell lines. Subsequently, we analyzed the alterations in telomerase activity, the length of TRF1s containing telomeric DNA, and the levels of intracellular p53 and p21WAF1/CIP1 of NHOK with different PDL numbers. Our data show that rapidly proliferating NHOKs, along with the immortalized and cancer cell lines, demonstrated readily detectable levels of telomerase activity. However, telomerase activity was barely detectable in NHOKs near or at senescence, although the TRF1 was maintained at a constant length in these cells. Further, unlike in fibroblasts, the intracellular p53 and p21WAF1/CIP1 levels in NHOKs were found to be diminished by the passage of cells. These data indicate that replicative senescence in keratinocytes from the human oral cavity may be associated with the loss of telomerase activity but not linked to an accumulation of p53 and/or p21WAF1/CIP1.

Results

The HPV-immortalized HOKs and Human Oral Cancer Cell Lines Demonstrate Telomerase Activity. To find out whether immortalized and oral cancer cells derived from human oral epithelial cells contain telomerase activity, we detected telomerase activity in an immortalized HOK cell line (HOK-16B; Ref. 36) and 10 human oral cancer cell lines using the PCR-based TRAP assay. The cell extract derived from telomerase-positive HN-12 cells provided by Oncor (Gaithersburg, MD) was used as the positive control, which produced the characteristic 6-bp ladder representing the addition of TTAGGG hexamers to the TRAP template. Pretreatment of the HN-12 extract with RNase A abolished the ladder, indicating that the ladder represents authentic telomerase activity. Telomerase activity was found in all tested cell lines and immortalized cells. Telomerase activity was detected at a level comparable to (HOK-16B-BaP, HOK-16B-BaP-T, SCC9, Tu-139, Tu-177, 183, and 1483) or slightly less than (SCC4 and Hep-2) that of HOK-16B (Fig. 1). These data demonstrate that cancer cells derived from the human oral cavity consistently possess a similar level of telomerase activity to the non-tumorigenic and immortalized HOK-16B cells with cloned HPV-16 genome, which showed a significantly high level of telomerase activity.

Proliferating NHOK Cells Contain a Significant Level of Telomerase Activity. To determine the presence of telomerase activity in replicating NHOKs, seven secondary NHOK cultures from different donors (ages ranging from 19 to 50) were analyzed. The protein extract derived from the HOK-16B cells and the same extract pretreated with RNase A were used as the positive and negative controls, respectively. A significantly high level of telomerase activity was detected in six NHOK cultures, although the activity in these NHOK cultures was notably lower than that in HOK-16B (Fig.
2) One NHOK culture contained only detectable telomerase activity. Telomerase activity was also detected from the epithelial cells separated from an oral epithelial tissue without in vitro culture (Fig. 3).

Cell Differentiation and Senescence of NHOK Are Induced by Serial Subcultures. To document the PDL numbers that induce replicative senescence of NHOKs, three primary NHOK cultures from different donors were continuously subcultured until they reached the postmitotic stage. The cultures were photographed after Giemsa staining, and the cells were collected to determine the level of telomerase activity. We observed some variation in the doubling time and cell proliferation potential among different cultures based on donor age (Table 1).

In one of the cultures (experiment 1), NHOK proliferated well until PDL 19, constituting the log phase of the growth curve (Fig. 4A). Cells maintained their squamous cell-shape morphology, although there was an alteration in the morphology as the cells underwent more doublings (Fig. 4B–D). Some NHOK cells showed signs of differentiation when they reached PDL 19 (Fig. 4E). However, the majority of the cells at this doubling level still maintained the replicating epithelial cell morphology. At PDL 20, most cells displayed the characteristics of terminal differentiation, showing highly enlarged and elongated cytoplasm, and terminally differentiated cells started to round up and detach from the culture dish (Fig. 4F). NHOKs past PDL 19 exited from the log phase of cell proliferation and apparently proliferated at a markedly reduced rate, as indicated in the mean doubling time: 25 h at PDL 9, 28 h at PDL 14, 52 h at PDL 16, 60 h at PDL 19, and 1204 h at PDL 20. Beyond PDL 20, there were not enough cells to establish the subsequent passage, and the serial subculture was stopped at this point. In other NHOK cultures
Table 1 Telomerase activity in NHOKs that were serially subcultured until replicative senescence

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* These numbers represent the relative intensities of telomerase activity in NHOK cultures of different PDLs with respect to that of the earliest NHOK culture (see "Materials and Methods").

(experiments 2 and 3), we recognized a similar pattern of terminal differentiation of NHOKs at PDL 26 (data not shown).

Telomerase Activity Is Lost in NHOKs That Undergo Replicative Senescence. The cells harvested at different PDL numbers were lysed with 1X CHAPS buffer, and TRAP assay was performed with 0.5 or 2.0 μg of the cell extract (Fig. 5A). The HOK-16B cells were used as the positive control among the immortalized and cancer cell lines because it is the immortalized counterpart of NHOKs (36). The HOK-16B cell extract treated with RNase A was included as the negative control. Significant telomerase activity was detected in the cell extracts obtained from the NHOK culture (experiment 1) at PDLs 9, 14, and 16 (Fig. 5A, Lanes 4–9; Table 1). These cultures demonstrated a similar level of activity to one another, although none of them showed nearly as much activity as HOK-16B. At PDL 19, however, the telomerase activity was almost undetectable compared to the cell extracts of rapidly proliferating NHOK, such that only the sample with the 2.0-μg extract produced a barely visible TRAP pattern (Fig. 5A, Lane 11). The loss of telomerase activity was further indicated in NHOKs at PDL 20 (Fig. 5A, Lanes 12 and 13), which is virtually identical to the RNase ATreated negative control.

This experiment was repeated twice with other NHOK cultures derived from different donors (Table 1). Similar to experiment 1, telomerase activity was significantly diminished by the increase in the number of PDLs, and it was barely detectable in cells near or at the senescent stage (Table 1). Telomerase activity was also determined in NHOFs isolated and cultured from gingival mucosa (Fig. 5B). A TRAP assay was performed with NHOF cell extract equivalent to 2.0 μg of protein as described above. As expected, NHOF cultures of passage 7, at which the cells were rapidly dividing, contained no functional telomerase. The above results provide evidence that proliferating NHOKs contain a significant level of telomerase activity that is subsequently lost during the course of their normal life span.

The Telomere Is Maintained at Constant Length in Serially Subcultured NHOKs. To correlate telomerase activity and telomere length in NHOKs, we determined the telomere length from NHOKs with different PDL numbers. Cellular DNAs extracted from NHOKs with PDLs 11, 14, 17, and 20 were digested with HinfI restriction enzyme. This enzyme restricts the cellular DNA at subtelomeric restriction sites of individual chromosomes, producing TRFs of varying length (10). After separating the digested DNA fragments by electrophoresis, TRF was detected by Southern hybridization with the 32P-labeled telomeric DNA probe containing (TTAGGG)16 (Fig. 6, A and B). The autoradiograph shows the characteristic smear of radioactive signal resulting from the variation of telomere length among different cells of the population and from the heterogeneity of the location of terminal restriction sites within the individual chromosomes of a given cell. The densitometric analysis of each smear showed that the signal intensity with respect to DNA length assumed a Gaussian distribution, and the mean TRFL was defined at the point of highest intensity as described previously (37). NHOKs with different PDL numbers tested contained the mean TRFL defined at ~6.0 kb, and shortening of telomere DNA was not detected with increasing PDL numbers. Two other NHOK cultures were serially subcultured until senescence, and TRFL analysis was performed as described above with the genomic DNAs isolated at PDLs 12, 14, 15, 17, 18, 19, and 20 for one NHOK culture (Fig. 6B, experiment 2), and PDLs 12, 14, 15, 17, and 18 for the other (Fig. 6B, experiment 3). The TRFL in both cultures remained within the range of 6.2–6.6 kb without detectable telomere shortening with respect to the increasing PDL numbers. Therefore, none of the three NHOK cultures tested for the TRFL analysis showed shortening of telomere DNA as the cells were serially passaged until they reached the postmitotic stage.

In parallel with the above experiment, we also determined the TRFL in NHOFs that were subcultured up to passage number 12. Genomic DNAs were extracted at passage numbers 6, 7, 8, 9, and 12 to determine TRFLs by Southern hybridization using the telomere DNA probe (Fig. 6, C and D). At passage 6, the mean TRFL of NHOFs, as indicated by the peak of the smear, was found to be ~11 kb, and the telomere DNA progressively decreased in length with each increasing passage number. Also shown in Fig. 6B is a nontelomeric band near ~1.8 kb (arrow), which maintained its size throughout the serial subculture of NHOFs, serving as an internal control. As shown in Fig. 6, C and D, the TRFL of NHOFs was gradually and significantly shortened by the subcultures. This result is in agreement with other reports that showed the shortening of telomere DNA with cellular aging (7, 11, 13, 38). Our data also show that telomere DNA of NHOFs shortened by ~178 bp per cell doubling because each passage represents, on the average, 2.8 cell doublings. The above results indicate that telomere DNA is maintained
Fig. 4. Primary NHOKs were serially subcultured until the cells reached replicative senescence. The total cell numbers at the beginning and end of each passage were used to determine the cell doubling time and PDLs, based on which cell proliferation curve was generated (A). The PDL numbers at which the cells were harvested are noted next to the data points on the plot. According to the proliferation curve, the log phase of cell proliferation was noted from PDL 0 to PDL 19, and cells apparently ceased to proliferate at PDL 20. The phase-contrast micrographs of the cells at PDLs 9, 14, 16, 19, and 20 demonstrate progressive changes in cell morphology during the serial subculture of NHOK (B–F). NHOKs maintained poorly differentiated squamous cell morphology at PDLs 9, 14, and 16 (B, C, and D, respectively). Characteristics of cell differentiation (i.e., highly stretched cell cytoplasm and enlarged nuclei filled with vacuoles) became clearly evident in NHOKs at PDLs 19 and 20 (E and F, respectively). Cells were fixed for 30 min with 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h and stained with 5% Giemsa stain for 5 min. Bar, 100 μm.

Fig. 5. TRAP assay of serially subcultured NHOKs. NHOKs cultured from an oral epithelial tissue was serially passaged until the cells reached replicative senescence. The cells harvested at PDLs 9, 14, 16, 19, and 20 were lysed in 1 × CHAPS buffer, and each cell extract containing 0.5 or 2.0 μg cellular protein (total extract amount is noted in the parentheses in the figure) was tested for the level of telomerase activity (A). HOK-16B, which is an HPV-immortalized counterpart of NHOKs, was included as the positive control, and the same cell extract was pretreated with RNase A as the negative control (Lanes 1 and 2, respectively). The TRAP pattern of NHOK indicates the presence of telomerase activity in cells of PDLs 9, 14, and 16 (Lanes 4–9), and a slightly higher level of the activity was detected with 2.0 μg of cellular protein than with 0.5 μg in cell extracts of each PDL. However, telomerase activity was not detected in cells at PDLs 19 and 20 (Lanes 10–13), except for the limited signal present at PDL 19 with 2.0 μg of cell extract (Lane 1). Telomerase activity in NHOFs at passage 7 was determined by TRAP assay with 2.0 μg of cellular extract as described above (B).

Serial Subculture Decreases the Intracellular wt p53 and p21WAF1/CIP1 Levels in NHOKs. Accumulation of intracellular wt p53 and p21WAF1/CIP1 has been reported in HDFs with increased cell doubling level (25, 27) and is believed to be an essential component responsible for the cell cycle arrest at G1 phase and for senescence (25, 29). Although a few reports showed no alterations in p53 level during replicative senescence of fibroblasts (34, 35), it has also been postulated that induction of wt p53 and p21WAF1/CIP1 in senescent HDFs is a consequence of accumulation of DNA damage, which results from telomere shortening beyond the critical length (25, 30). The linkage between the induction of p53 and/or p21WAF1/CIP1 and the senescent phenotype of cells has been reported by other studies (29, 31, 32). Be-
cause telomere shortening is not observed in serially subcultured NHOKs, we asked whether the activation of a DNA damage pathway as determined by the induction of wt p53 and/or p21WAF1/CIP1 levels could still be observed in senescent NHOKs in the absence of telomere shortening.

To answer this question, the cellular levels of wt p53 and p21WAF1/CIP1 were determined from NHOKs with different PDL numbers by Western blot analysis (Fig. 7A and Table 2). The cellular differentiation and proliferation status of NHOKs at different PDLs were determined by comparative analyses of the intracellular protein levels of involucrin and PCNA, respectively. The protein level of involucrin, which constitutes the precursor of the protein component of the cornified envelope in differentiated keratinocytes (39), was successively increased in NHOKs of higher PDLs. In contrast, the level of PCNA, known as the DNA polymerase processivity factor, which is strongly associated with cellular proliferation (40, 41), was rather notably diminished during serial subculture of NHOKs. In the same cellular extracts as those used to determine involucrin and PCNA levels, the protein levels of both p53 and p21WAF1/CIP1 were found to be progressively decreased in the NHOK cultures of increasing PDLs. The
Table 2  Intracellular levels of p53 and p21WAF1/CIP1 in NHOKs that were serially subcultured until replicative senescence

Western blot analysis was performed with 100 µg of total cellular protein separated by 10% SDS-PAGE. Each experiment represents an independent Western blot analysis using different NHOK cultures. The NHOK cultures used in experiments 1 and 2 reached senescence at PDL 20 and that of experiment 3 at PDL 27. NHOK cultures used in Western blot analyses correlate to those of the TRAP assay (see Table 1), except for experiment 2. Western analysis of experiment 3 is shown as the representative figure (Fig. 7A).

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a These numbers represent the normalized intensities of the bands obtained in NHOK cultures of different PDLs with respect to that of the earliest NHOK culture (see "Materials and Methods").
b ND, not detected.

...cellular p53 and p21WAF1/CIP1 protein levels were determined in two other NHOK cultures, which were serially subcultured until senescence (Table 2). In both cultures, we also observed a progressive decrease in the levels of p53 and p21WAF1/CIP1 with increasing PDL numbers. Further, the quantity of p53 in rapidly proliferating NHOKs at PDL 21 (experiment 3) was compared with those of NHOF cells at passages 6, 7, 8, 9, and 12 (Fig. 7B). The p53 level in NHOFs was notably lower than that of proliferating NHOKs, and it was not altered by subcultures.

The reduction in the p53 level in late-passage NHOKs was further indicated by in situ immunofluorescence staining with anti-human p53 monoclonal antibody. An NHOK culture that is different from those used for the Western blot analysis was continuously passaged, and the relative intensity of the fluorescent staining of p53 was compared between cultures at PDL 19 (Fig. 8B) and at PDL 14 (Fig. 8A). The bright staining of p53 in early-passage NHOKs was highly localized in the nuclei, whereas the late-passage population was predominated by the cells with faint and diffuse staining of p53 throughout the entire cytoplasm. Therefore, three independent Western blot analyses using different NHOK cultures as well as the immunofluorescence staining for intracellular level of p53 consistently demonstrate the decline in the levels of p53 and of p21WAF1/CIP1 in NHOK cultures that are progressing toward replicative senescence. The above results provide evidence that the accumulation of intracellular p53 and/or p21WAF1/CIP1 is not observed during the senescence of NHOKs and suggest that these proteins may not be involved in the process of senescence in NHOKs.

Discussion

Our study demonstrates that the immortalized HOK-16B cell line and all tested human oral cancer cell lines contain significantly high levels of telomerase activity. These results are in agreement with other studies that show the presence of telomerase activity in most primary human malignant cells, cancer cell lines, and tissues (24). Although our study does not provide direct evidence, the presented data support the hypothesis that up-regulation of telomerase activity is necessary for the maintenance of the phenotypes of immortal...
ized and cancer cells and is required for the conversion of normal cells to immortalized or malignant phenotypes (12, 20, 21). Therefore, the development of specific inhibitors of telomerase, although there are some concerns, may aid the treatment of cancer.

Our data also show that rapidly proliferating NHOK cultures contain significant telomerase activity. One could, however, argue that, like fibroblasts, proliferating NHOK may not contain telomerase, and that telomerase activity detected in NHOK cultures may result from the consequence of \textit{in vitro} cultures of cells because of the abundant presence of growth factors in the culture medium. To rule out this possibility, we determined the enzyme activity in an epidermal tissue separated from an oral tissue specimen. We were able to detect enzyme activity in the oral epidermis, although activity in the tissue was notably lower than that in secondary cell cultures. Lower telomerase activity in the epidermis compared to NHOK cultures may be due to the presence of both rapidly proliferating and nonproliferating keratinized cells in the epidermis, whereas secondary NHOK cultures contain mainly proliferating cells. Although most somatic human cells do not exhibit this enzyme activity (14, 21, 22, 24), the presence of telomerase in proliferating NHOK cultures and the oral epidermis is not surprising. In fact, previous reports showed that the swiftly proliferating basal cell population of normal human foreskin epidermis demonstrated telomerase activity, whereas the nonproliferating suprabasal population of the epidermis does not show this activity (19, 23). These observations suggest that telomerase may be necessary for the proliferation of normal human epithelial cells, including NHOKs.

Inasmuch as nonproliferating suprabasal cells of normal epidermis do not demonstrate telomerase activity, we predicted that the down-regulation of telomerase activity in the suprabasal cells might be associated with replicative senescence of epithelial cells. To test this assumption, we subcultured a primary NHOK culture until they reached the postmitotic stage and determined the activity of cells with different numbers of PDLs. As predicted, rapidly proliferating NHOK cultures demonstrated a significant level of telomerase activity. However, the NHOK cultures near senescence, although they still proliferated, showed markedly reduced enzyme activity. Further loss of telomerase activity was indicated in the cultures in which cells completely ceased to proliferate. This experiment was repeated using two additional NHOK cultures. Although the proliferative potential of individual NHOK culture showed some variation among one another, we always observed the strong telomerase activity associated with rapidly proliferating NHOK cultures and a rather rapid loss of telomerase activity in cultures near and at replicative senescence. These results are in agreement with previous reports that showed that telomerase activity correlates to the proliferative activity of cells (42–45). The present study, however, provides the first experimental evidence that senescence of human epithelial cells induced by continuous subculture is associated with the loss of telomerase activity, and that functional telomerase may be necessary for the proliferation of HOKs. Furthermore, NHOK cultures near senescence with drastically reduced levels of telomerase activity were found to undergo limited cell doublings before cells completely lost their potential to divide.

The present model of cellular senescence based upon the telomere hypothesis suggests that telomere attrition beyond the critical length (i.e., the minimum length of telomere required for continued cell division) following each round of DNA replication results in senescence (46). Construction of this model was based upon the original observations that telomere shortening accompanies cell doublings (47) and that normal HDFs have finite proliferative capacity (48). Subsequently, the association between replicative senescence and shortening of telomere length has been well documented in normal somatic cells (7–10, 13, 38, 49). However, our results demonstrated that the mean TRFL remained constant at ~6.0 kb in NHOK cells that were serially passaged until the cells became postmitotic. The TRFL in senescent NHOKs is significantly greater than those of fibroblasts at senescence (~2.8 kb; Ref. 7) and human embryonic kidney cells transformed by SV40 (4 kb) at crisis (10).

The possibility that telomere attrition cannot be detected in NHOKs due to the limited cell life span has been ruled out because TRFs of NHOFs, telomerase-negative primary human fibroblasts of oral mucosa, were found to shorten considerably during serial subculture. Telomere shortening observed in each successive subculture of NHOF represents ~0.5 kb per passage and 178 bp per doubling because cells underwent 2.8 doublings per passage. Because the TRFL analysis for NHOKs was performed over the spectrum of nine cell doublings, we conclude that the sensitivity of the Southern hybridization technique would be sufficient to detect telomere shortening in NHOKs if there were indeed shortening of telomeres. The lack of telomere attrition in NHOKs can be clearly explained by the finding that rapidly proliferating NHOKs contain a significant level of telomerase activity. Unlike activated human T lymphocytes, in which telomere DNA shortens despite the presence of functional telomerase (43), the telomerase activity in NHOKs may be sufficient to maintain telomere length during the normal life span of cells in this culture. Although telomerase activity was significantly reduced in NHOK cultures immediately preceding the senescent culture, detectable telomere shortening is not expected, even near the end of the replicative life span, because the cells apparently undergo limited cell doublings before the cells completely lose their potential to divide. The above results suggest that the telomere length in NHOKs may not be a reliable marker for the proliferative capacity of the cells in culture.

Numerous reports have indicated that replicative senescence of normal human fibroblasts may result from the enhanced intracellular wt p53 and p21\textsuperscript{WAF1/CIP1} protein levels (25, 28). This hypothesis is based on the observations that the levels of these proteins are elevated in senescent fibroblasts, and that disruption of p21\textsuperscript{WAF1/CIP1} in HDFs causes bypass of senescence (25, 28, 29). To study whether the senescence of NHOKs is associated with the elevation of p53 and p21\textsuperscript{WAF1/CIP1} in cells, we determined the levels of these proteins in NHOKs with different PDL numbers. In contrast to fibroblasts, continuous subculture of NHOKs until the cells reached replicative senescence significantly de-
creased the amount of p53 and p21WAF1/CIP1 in cells. A total of four NHOK cultures obtained from different donors were subcultured, and the decline of the levels of p53 and p21WAF1/CIP1 with respect to the PDL numbers was observed in all NHOK cultures. A parallel study involving serially subcultured NHOF cultures revealed no significant decrease in the level of p53 in cells. In fact, proliferating NHOF cultures contained barely detectable levels of p53, which remained unchanged throughout the subcultures.

Although the detailed modes of action remain unknown, these data suggest that replicative senescence of NHOKs is associated with the loss of telomerase activity but is not linked to the accumulation of wt p53 and p21WAF1/CIP1. Our results apparently contrast to the senescence model proposed by Vaziri and Benchimol (25), who stressed the importance of the activation of cyclin-dependent kinase inhibitor (e.g., p21WAF1/CIP1 and p16INK4a), be it p53 dependent or independent, during the senescence of HDFs. This contradiction may stem, in part, from the fact that significant telomerase activity is present in rapidly proliferating NHOKs, whereas it is absent in skin fibroblasts (19, 24). The data presented here point to the conclusion that telomerase activity present in proliferating NHOKs maintains the telomere at a constant length and thus prevents the accumulation of DNA aberrations that may otherwise occur in telomerase-negative fibroblasts as the telomeric sequences shorten beyond the critical length. In the absence of DNA aberrations during the senescence of NHOKs, the DNA damage pathway involving p53 and/or p21WAF1/CIP1 may remain silent, and a novel mechanism of cellular senescence that is fundamentally different from the current model may ensue.

Materials and Methods

Tissue Specimens. Human oral mucosal tissue was obtained from patients (ages ranging from 19 to 50) undergoing oral surgery. The tissue samples were first thoroughly washed three times with calcium-free MEM (Life Technologies, Inc., Grand Island, NY) supplemented with 15 ml/g ml gentamicin (Gemini Bio-Products, Calabasas, CA) and treated with calcium-free MEM containing collagenase (type I; 150 units/ml; Sigma Chemical Co., St. Louis, MO) and dispase (grade II; 2.4 mg/ml; Boehringer-Mannheim, Indianapolis, IN) for 90 min at 37°C in 95% air and 5% CO2 to separate the epithelial layer from the underlying connective tissue. The separated epithelial layer was used either for a cell culture or for obtaining cell lysate without culture. The cell lysate was obtained by first quickly freezing the tissue with liquid N2 and grinding it in a tissue grinder (Belco Glass, Vineland, NJ). The lysate was then resuspended in 100 ml of 1 CHAPS lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM EDTA, 0.5% 3-[3-cholamidopropyl]dimethylammonium]-1-propanesulfonate, 10% glycerol, 5 mM ß-mercaptoethanol, 1 mg/ml pepstatin, 10 mg/ml leupeptin, and 0.1 mM phenylmethylsulfon fungluconite] incubated on ice for 30 min, and centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was quickly frozen with liquid N2 and stored at -80°C for TRAP analysis.

Cells and Cell Culture. Primary NHOKs were prepared from separated epithelial tissue and cultured in keratinocyte basal medium containing 0.15 ml calcium and supplementary growth factor TBS buffer (10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM EDTA, 0.5% 3-[3-cholamidopropyl]dimethylammonium]-1-propanesulfonate, 10% glycerol, 5 mM ß-mercaptoethanol, 1 mg/ml pepstatin, 10 mg/ml leupeptin, and 0.1 mM phenylmethylsulfon fungluconite] incubated on ice for 30 min, and centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was aliquoted and frozen in liquid nitrogen for TRAP assay and for the determination of protein concentration. The TRAP reaction was performed with positive control cell extract (HN-12 from Oncor, Inc.) containing telomerase activity and negative control HN-12 extract treated with 20 ml/ml RNase A (Sigma Chemical Co., St. Louis, MO).

Telemorase Reaction Mixture was prepared by adding 2 ml of cell lysate containing 0.5 or 2.0 g/ml of cellular protein to 48 g/ml of solution comprising 1 TRAP reaction buffer [20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 63 mM KCI, 0.005% Tween 20, and 1 mM EGTA], 50 mM each deoxynucleotide triphosphate, 0.05 g/ml of T5 primer end-labeled with 20 µCi of [γ-32P] dATP (4500 Ci/mmol; ICN, Costa Mesa, CA), 1 ml primer mix, and 0.4 unit Taq polymerase (Perkin-Elmer, Foster City, CA). The mixture was incubated at 30°C for 30 min, and telomerase reaction product was amplified using a DNA Thermal Cycler (Perkin-Elmer). The following conditions were used for the PCR cycle: 30 cycles at 94°C for 30 s and 55°C for 30 s, followed by one delayed extension cycle at 72°C for 10 min. The PCR products were run in 12.5% nondenaturing polyacrylamide gel in 1 Tris-borate EDTA for 90 min at 4°C. After the gels were stained with silver nitrate, the radioactive signal was detected by autoradiography. For the TRAP assay of the NHOK cultures that were serially subcultured, the resulting radiographs were scanned, and the intensity of the bands that were separated by six bases per lane were collectively measured for densitometric analysis using NIH Cell Growth & Differentiation
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Image version 1.60 (NIH, Bethesda, MD). The relative intensity of telomerase activity with respect to that of the earliest NHOK culture was obtained by subtracting the background intensity from that of NHOK cultures at different PDLs and subsequently by dividing them by that of the earliest NHOK culture.

Telomere Length Determination by Southern Blot Hybridization. Genomic DNAs isolated from three different NHOK cultures at different PDLs or from one NHOF culture of different passage numbers were extracted by the conventional method as described previously (36). Ten μg of DNA digested with restriction enzyme HindIII were electrophoresed in a 0.8% agarose gel and transferred to Hybond nylon membrane (Amer sham, Arlington Heights, IL). After rinsing the filter with 2× SSPE, the filter was baked at 80°C for 2 h in a vacuum oven. The filter was soaked in 2× SSPE for 5 min and prehybridized in 20 ml of prehybridization buffer (0.5 M NaH2PO4 (pH 7.2), 7% SDS, 1% BSA, and 0.5 mM EDTA) at 65°C for 4 h. Hybridization was performed with 32P-labeled telomere probe (see below) in the above buffer at 65°C for 16–24 h. Subsequently, the filter was washed with 2× SSPE-0.1% SDS at room temperature for 15 min and with 0.1× SSPE-0.1% SDS twice at 65°C for 15 min each. The radioactive signals were detected by autoradiography, and densitometric analysis of each lane was performed using NIH Image version 1.60 (NIH). Because the densitometric profiles assumed a Gaussian distribution, the mean TRF was defined at the length of DNA that corresponded to the peak of each signal as described previously (37).

The telomeric DNA probe was obtained from the PCR products of a TRAP assay. The PCR products were purified from 2% agarose gel using the Qiagen Gel Extraction kit (Qiagen, Inc., Chatsworth, CA) and cloned into a pCRll vector (Invitrogen, San Diego, CA). After restriction digestion with EcoRI, the cloned plasmid yielded a 100-bp fragment comprising 16 YTAGG repeats flanked by the 5′ and 3′ primer sequences. The isolated restriction fragment was labeled with [γ-32P]ATP (ICN) using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN).

Western Analysis. Three different NHOK cultures and one NHOF culture harvested at each passage were lysed, and the cell extracts were processed for Western analysis to determine the intracellular levels of p53, p21WAF1/CIP1, PCNA, and involucrin using Western-Light kit (Tropic, Inc., Bedford, MA) as described previously (50). Monoclonal antibodies to p53 (pab1801), p21WAF1/CIP1 (E1A10), and PCNA were obtained from Oncogene Research Products (Cambridge, MA), and an antibody against involucrin from Sigma Corp. (St. Louis, MO). After probing with the respective antibodies, the membrane was stained with 1× Ponceau S stain for 10 min to reveal the total protein amount loaded per each lane. The bands resulting from Western analysis as well as the Ponceau S staining were scanned, and densitometric values were obtained using NIH Image version 1.60 (NIH). Normalization of the signal intensity with respect to the quantity of the loaded protein per each lane was performed by multiplying the densitometric values of NHOK cultures at different PDLs, from which the background intensity was subtracted, by those of the corresponding Ponceau S staining. The normalized values of each lane were subsequently divided by that of the earliest culture of NHOKs to determine the relative intensity of the successive culture of NHOKs.

Immunofluorescence Analysis. Cells grown in tissue culture chambers (Nunc, Inc., Naperville, IL) were washed with cold 1× PBS, fixed in acetone and methanol (1:1, v/v) at −20°C, and processed for immunofluorescence staining. Essentially, cells were incubated with mouse anti-human p53 monoclonal antibody (pAb1801) diluted to 6 μg/ml in 1× PBS with 0.2% gelatin for 60 min at 37°C, washed extensively with a solution containing 1× PBS, 0.2% gelatin, and 0.05% Tween 20 (washing buffer), stained with affinity-purified fluorescein-conjugated goat anti-mouse antibody (1:100 dilution; Cappel Organon Teknika, Durham, NC) for 60 min, and rinsed again extensively with the washing buffer. Finally, samples were mounted with a gelvatol-glycerol solution containing 2.5% 1,4-diazobicyclo-(2.2.2) octane (Sigma) and examined with a transmission epifluorescence microscope with the appropriate filter and optics.

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