Effects of Telomerase and Viral Oncogene Expression on the In Vitro Growth of Human Chondrocytes

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Senescent chondrocytes accumulate with aging in articular cartilage, a process that interferes with cartilage homeostasis and increases the risk of cartilage degeneration. We showed previously that chondrocyte telomere length declines with donor age, which suggests that the aging process is telomere dependent. From these results we hypothesized that telomerase should delay the onset of senescence in cultured chondrocytes. Population doubling limits (PDL) were determined for chondrocytes expressing telomerase. We found that telomerase alone did not extend PDL beyond controls that senesced after 25 population doublings. The human papillomavirus 16 oncogenes E6 and E7 were transduced into the same cell population to investigate this telomere-independent form of senescence further. Chondrocytes expressing E6 and E7 grew longer than the telomerase cDNA (hTERT) cells but still senesced at 55 population doublings. In contrast, chondrocytes expressing telomerase with E6 and E7 grew vigorously past 100 population doublings. We conclude that although telomerase is necessary for the indefinite extension of chondrocyte life span, telomere-independent senescence limits PDL in vitro and may play a role in the age-related accumulation of senescent chondrocytes in vivo.

The potential for human chondrocyte proliferation declines, and senescent cells accumulate with aging in articular cartilage (1). Based on examples in other tissues, it has been suggested that these cellular changes contribute to osteoarthritis, a common and costly age-related degenerative disease of cartilage (2–4). Senescence is induced by multiple mechanisms that can often be traced to different environmental conditions; thus it may be possible to devise strategies to prevent or delay the onset of senescence in cartilage or other tissues. With this as a long-term goal, we undertook the present study to better define the cellular mechanisms that regulate chondrocyte growth and senescence.

Senescence is readily observed in vitro in long-term cultures of continuously dividing somatic cells, which enter growth arrest after a characteristic number of population doublings (PD) (5). This limit approaches 60 PD for human fibroblasts, but chondrocytes may be limited to 30–35 PD (6,7). Growth arrest at this stage is referred to as mortality stage 1 (M1) (8). M1 growth arrest is induced by replication-dependent loss of telomeres, the DNA structures found at chromosomal termini that are necessary for DNA replication (9). Telomere sequences are lost with each round of DNA synthesis and may eventually erode to a length that can no longer support chromosomal replication (10–12). Telomere erosion is an inevitable consequence of mitosis in all cells; however, net losses can be avoided through the activity of telomerase, a DNA polymerase that replaces telomeric sequences as they are lost (13). Telomerase is active in most immortal cell lines, including germ cells and cells derived from tumors (14–16). Moreover, normal fibroblasts transfected with the telomerase cDNA (hTERT) are effectively immortalized without the phenotypic disruption associated with oncogene transfection (17–19). This suggests that the enzyme extends PD limits solely through the maintenance of telomeres.

Although hTERT is sufficient to immortalize fibroblasts, some cell types, such as keratinocytes and mammary epithelial cells, have been found to be refractory to immortalization by hTERT alone (20,21). These data imply that PD can be limited independently of telomere erosion. Human keratinocytes and epithelial cells stop growing in vitro at low PD by means of a mechanism that is distinct from M1 growth arrest. The causes of this form of arrest, termed mortality stage 0 (M0), are not clear; however, oxidative stress and other suboptimal environmental conditions appear to play a role (22,23). Introduction of the high-risk human papillomavirus 16 (HPV16) oncogene E7 allowed epithelial cells to overcome M0 arrest and to continue proliferation to M1. This is thought to occur because E7 blocks the activity of the p16/pRb pathway that induces M0 arrest (24). A second oncogene, E6, targets the p53 pathway and may also play a role in overcoming M0 (22,25). E6 and E7 coexpression is sufficient to immortalize epithelial cells, but this depends on reactivation of telomerase by E6, an effect that does not occur in fibroblasts (26). These studies demonstrate that at least two distinct mechanisms lead to growth arrest in somatic cells and show that the requirements for immortalization differ in different types of cells.

Cartilage aging studies in our laboratory suggested that chondrocytes senesce in vivo by means of a telomere-depen-
dent mechanism. This implies that chondrocytes reach their population doubling limit (PDL) as a consequence of M1 growth arrest. From this, we hypothesized that a constitutive expression of telomerase would be sufficient to immortalize these cells. To test this hypothesis we transduced chondrocytes with the hTERT cDNA and determined the effect of telomerase expression on the population doubling limit (PDL) of the cells. This experiment showed that hTERT did not extend the PDL, as both vector controls and hTERT transductants senesced after ~25 PD, indicating that chondrocyte population growth was arrested at M0 rather than at M1. Because E6E7 transduction overcomes this barrier in other cell types, we tested the effects of these proteins on chondrocyte growth. We found that E6E7 alone extended the PDL to ~60 and that the combination of E6E7 and hTERT allowed growth to at least 100 PD. These data indicate that in vitro chondrocyte growth is limited by telomere-independent mechanisms that may contribute to the age-related accumulation of senescent chondrocytes in cartilage.

**METHODS**

**Cell Culture**

Human articular cartilage was harvested from the central portions of the medial and lateral surfaces of a tibial plateau removed from a 47-year-old patient undergoing total joint replacement for degenerative joint disease. Chondrocytes were isolated as described (1) from approximately 4 g of cartilage and plated in primary monolayer cultures in Dulbecco’s modified Eagle medium with 10% fetal calf serum (Life Technologies, Rockville, MD). This initial population was grown for two passages (1:2 split) and then seeded into 60-mm dishes at a density of ~200,000 cells/dish.

**Viral Transduction**

A cDNA encoding hTERT (Geron Corporation, Menlo Park, CA) was inserted as an EcoRI fragment into the retroviral vector pLXSN. Chondrocytes (third passage) were transduced with pLXSN or pLXSN containing the hTERT cDNA. After 24 hours of exposure the viral infection medium was replaced with fresh medium containing G418 (200 µg/ml) for selection of positive transductants. The transduced cells were then grown for 2 weeks in G418 before reverting to nonselective medium. E6 and E7 transductants were carried out as described previously (27).

**Southern Blot Analysis for Mean Terminal Restriction Fragment Length**

DNA was isolated from ~1 × 10^6 to 5 × 10^6 cells by using a DNEasy kit (Qiagen, Valencia, CA) according to the manufacturer’s directions. Southern blots were performed essentially as described (13), except that nonradioactive methods were used to detect telomere sequences according to Genius system directions published by the manufacturer (Roche, Indianapolis, IN). The probe was a synthetic oligonucleotide complementary to human telomere repeat sequences [(CCCTAA)_4] (22) and labeled at the 3’ end with digoxigenen (Sigma Genosys, St Louis, MO). An antidigoxigenen alkaline phosphatase-conjugated antibody and a chemiluminescent substrate, CDP-Star (Roche), were used to detect the digoxigenen-labeled probe. All DNA samples were digested and analyzed on at least two gels.

**Telomerase Activity Assay**

Telomerase activity was determined by using a telomere repeat amplification protocol (TRAP) assay kit (Roche) according to the supplier’s directions. This version of the assay allows relative quantitation of telomerase activity if extracts are prepared from equal numbers of cells. Accordingly, we used a standard number of cells (1 × 10^6) for each determination. Relative telomerase activity (RTA) was calculated as follows: [(OD_sample − OD_negative control)/OD_internal standard]/[(OD_positive control extract − OD_extract buffer)/OD_internal standard].

**PCR Analysis for E6 Expression**

The polymerase chain reaction (PCR) was used to confirm expression of E6 RNA. Total RNA was extracted from ~1 × 10^6 cells by using an RNase kit (Qiagen) according to the manufacturer’s directions. One microgram of total RNA was reverse transcribed by using a Smart cDNA synthesis kit (Clontech, Palo Alto, CA) and the resulting cDNA preparation was amplified by using E6 sequence-specific primers (HPV E6 forward, 5’GCCGCCGAAAAGTTACACAG3’; HPV E6 reverse 5’GCAACAGAGCTAGTCGAGC3’) that were designed to amplify a 368 base pair product. PCR conditions for E6 amplification were as follows: 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 40 seconds, and one cycle of 72°C for 5 minutes. Glyceraldehyde phosphate dehydrogenase (GAPDH)-specific primers and PCR conditions were described previously (1). PCR products were fractionated on agarose gels, stained with ethidium bromide, and photographed.

**Western Blot Analysis for E7 Expression**

A Western blot analysis was performed by using extracts from subconfluent cultures. Cells in 100-mm dishes were extracted in 0.25 ml of lysis buffer, which consisted of 25 mM Tris-HCl pH 7.5, 125 mM NaCl, 2.5 mM ethylenediamine tetra-acetic acid (EDTA), 0.05% sodium dodecyl sulfate (SDS), 0.5% nonidet P-40, 0.5% deoxycholate, and 10% glycerol, containing protease and phosphatase inhibitors. Total protein concentration was determined by bichinchoninic acid assay using a commercial kit (Pierce, Rockford IL). Forty micromgrams of total protein in reducing buffer was fractionated on a 14% SDS–polyacrylamide gel using a discontinuous buffer system. Western blots were performed by using Immobilon-P nylon membranes (Millipore, Bedford, MA). The blots were probed with the mouse monoclonal antibody directed against HPV16-E7 (clone 8C9) as described by the supplier (Zymed, San Francisco, CA). Blots were probed again with a goat–antimouse alkaline phosphatase conjugated secondary antibody (Promega, Madison, WI), developed in CDP-Star (Roche), a chemiluminescent substrate, and exposed to Biomax MR autoradiography film (Eastman Kodak, Rochester, NY) for 30 minutes.

Karyotypic analysis of the hTERT/E6E7 population was performed to determine if normal chromosomal structure was maintained. The analysis was performed by standard cytogenetic methods on 10 cells harvested at 65 PD.
RESULTS

Chondrocytes were grown continuously in monolayer culture over a period of several months to determine the effects of hTERT and E6E7 transduction on PDL. PD was calculated each time the cultures were trypsinized and passed to new flasks. Accumulated PD was plotted as a function of time to show changes in population growth rates that occurred with successive passages (Figure 1). The graph shows that chondrocytes transduced with the retroviral vector alone (LXSN) stopped growing after 25 PD. The growth of chondrocytes transduced with the virus bearing hTERT was also limited to 25 PD, indicating that telomerase expression did not extend the life span of this population. In contrast, E6E7 transduction extended growth to ~55 PD, indicating that although viral oncogene expression was not sufficient for immortalization, it allowed cells to bypass early growth arrest at 25 PD. The population transduced with both hTERT and E6E7 surpassed even the 55 PD limit and continued to grow past 100 PD with no apparent decline in growth rate. These data indicate that two different mechanisms limit population growth in chondrocytes: an early, telomerase-independent limit that was overcome with the introduction of viral oncogenes, and a later telomerase-dependent limit.

Mean telomere restriction fragment analysis was performed to determine if hTERT transduction maintained telomere length in growing chondrocyte populations. The Southern blot shown in Figure 2 illustrates the results of this analysis. The blot shows that extensive telomere erosion occurred in LXSN cells between 10 PD and 20 PD when population growth rate began to decline. Similar losses occurred in E6E7-transduced cells between 12 PD and 40 PD. Erosion continued to at least 50 PD, near the point of growth arrest. The introduction of hTERT lead to markedly different results: telomere length was maintained through population doublings in both the hTERT and the hTERT/E6E7 populations. Moreover, telomere lengths were always
greater in both these populations than in LXSN or E6E7 transductants. These results show that hTERT transduction prevented replication-dependent telomere erosion, indicating that telomerase expression levels were sufficient to maintain telomere length.

The TRAP assay was used to confirm that telomere maintenance was associated with telomerase activity in hTERT transductants. The assay was performed with extracts from subconfluent cultures harvested at successive PD. Equal numbers of cells from each population were used for each extraction and the results were normalized to a standard, telomerase-positive extract provided by the manufacturer. RTAs calculated from these data are shown in Figure 3. This histogram reveals that telomerase was highly active in both populations carrying the hTERT gene. Activity levels in hTERT cells did not decline from 14 PD to 20 PD (right column). Activity in hTERT/E6E7 was stable from 14 PD to 51 PD (right column), indicating that hTERT transgene expression was very stable over time. Although telomerase expression is induced by E6 in keratinocytes and epithelial cells, activity remained at background levels in E6E7 chondrocytes from 14 PD to 50 PD, indicating the absence of such effects in this cell type. These results clearly link telomere maintenance with telomerase activity and with the presence of the hTERT transgene.

A reverse transcription PCR (RT-PCR) and Western blot were used to confirm E6 and E7 expression, respectively. Results of the RT-PCR analysis are shown in Figure 4. A 368 base pair band corresponding to the expected E6 product was amplified from the E6E7 and hTERT/E6E7 lines, but not from the hTERT or LXSN lines. The positive control GAPDH reactions yielded a 272 base pair product in all four lines. A Western blot analysis for E7 protein expression is shown in Figure 5. Immunoreactive bands of the expected molecular weight (~15 kDa) were prominent in the E6E7 and hTERT/E6E7 lines but were absent in both the hTERT and LXSN lines. The nonspecific signals from higher molecular weight proteins were similar in strength in all four lanes, indicating that similar amounts of protein were loaded in each lane. Together these experiments confirm expression of E6 and E7 in the appropriate cell lines.

A cytogenetic analysis of hTERT/E6E7 cells at 65 PD revealed a number of chromosomal abnormalities. Chromosome numbers ranged from 43 to 47. A composite karyotype based on abnormalities common to 6 of 10 cells indicated a deletion of the long arm of chromosome 5 with a breakpoint at 5q13; 2. An additional abnormality in at least 2 of 10 cells was an addition of unknown material to the long arm of chromosome 19 at 19q13.4; 3.

**DISCUSSION**

The results of this study showed that hTERT transduction was not sufficient to extend the life span of a normal chondrocyte population under typical monolayer culture conditions. We found that although hTERT was expressed at sufficient levels to maintain telomere length in dividing chondrocytes, the population nevertheless stopped growing after only 25 PD, the same growth limit we observed in controls lacking telomerase. These data showed that M0 growth arrest at 25 PD was induced by mechanisms unrelated to telomere length. Cells carrying the viral oncogenes E6 and E7 overcame the 25 PDL, doubling life span to 55 PD. Southern blots showed extensive telomere erosion over this period, indicating that cells carrying E6E7 were still subject to M1 growth arrest by means of replicative senescence. In-
definite growth was only achieved when hTERT was coexpressed with E6E7, indicating that telomerase was sufficient to overcome M1 arrest in chondrocytes.

Although E6E7 appeared to extend PDL, we did not find evidence that the E6 protein induced endogenous telomerase expression as has been reported for keratinocytes and epithelial cells (26). This finding prompted us to confirm that the oncogenes were expressed in the appropriate cell lines. We found that E6 RNA and E7 protein were detectable in both lines transduced with viral DNA, indicating that chondrocytes, like fibroblasts, do not reactivate hTERT expression in the presence of E6.

In conclusion, we found that chondrocytes senesce in vitro in two stages: an early telomere-independent stage, and a later telomere-dependent stage. Early senescence sharply limited growth to approximately half the replicative potential of the population. The fact that this block was alleviated by oncogene expression suggests that arrest was caused by activation of one or more tumor suppressor proteins that mediate responses to DNA damage (22,28–30).

The relevance of this finding to cartilage aging and degeneration are unclear, because damage may be caused postisolation by culture-related factors such as oxidative stress. Although this would seem to suggest that early senescence is a culture artifact, oxidative conditions in articular cartilage could also cause sufficient DNA damage to provoke senescence in vivo. Future studies will help to determine if this form of growth arrest contributes to the accumulation of senescent chondrocytes with cartilage aging.

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Editor Nominations

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