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Human telomerase reverse transcriptase (hTERT) extends the lifespan of canine chondrocytes in vitro without inducing neoplastic transformation

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Abstract

To determine if the exogenous expression of the human telomerase reverse transcriptase (hTERT) protein can extend the in vitro lifespan of chondrocytes from normal and osteoarthritic canine donors, articular chondrocytes were harvested and expanded initially in monolayer culture. Cells were transfected with pCIneo or pCIneo–hTERT and selected using G418. Transfectants were cultured either in monolayer or alginate beads and telomerase activity, replicative lifespan and the tumourogenic potential of the transfected cells were assessed. hTERT expression in canine chondrocytes prolonged the replicative lifespan of these cells but did not permit growth in low serum conditions or promote the formation of foci in anchorage independence assays. In addition, hTERT expression resulted in the down-regulation of MMP-1. This suggests that hTERT may represent a tool for the generation of tissue engineered chondrocytes suitable for autologous re-implantation into the affected areas of osteoarthritic joints.

Keywords: Osteoarthritis; Chondrocyte; Senescence; Telomere; Telomerase

Introduction

The relationship between increasing age and the accelerated pathogenesis of osteoarthritis (OA) has been well documented, and although increasing age is not a definitive factor in the onset of OA, the fact that the severity of the disease escalates in older patients has been evident for some time (Martin and Buckwalter, 2002a; Mollano et al., 2002). Cellular ageing is a complex process and in human cells there is evidence associating increasing age with a reduction in proliferative potential, alterations in gene expression and the progression of the cells into an irreversible state known as replicative senescence (Campisi, 1997).

The mechanism of replicative senescence is partly controlled by telomeres which comprise several kilobases of tandem TTAGGG repeats bound by specific proteins which cap the extreme 3′ ends of chromosomes. In most somatic cells, telomeres shorten at each cell division (Levy et al., 1992) and when the telomeres reach a certain threshold limit, the cells enter senescence and are unable to undergo further cell divisions (Hayflick, 1965). Although originally reported as an in vitro phenomenon it is now known to occur as an important in vivo event. In OA, premature chondrocyte senescence, often the result of oxidative stress and compounded by trauma and/or inflammation, is associated with the increased production of catabolic factors that result in the degradation of the complex cartilage extracellular matrix (Chen, 2000; Martin and Buckwalter, 2002b; Price et al., 2002). In the majority of tumour cells, germ cells, stem cells and in a small

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proportion of somatic cells, telomere length is maintained by the action of the enzyme telomerase (for review, see Bryan and Cech, 1999).

It has previously been demonstrated that the exogenous application of the catalytic component of human telomerase (hTERT) prevents telomeric attrition in several different types of human and animal cells, and indeed this approach has previously been examined in human chondrocytes (Bodnar et al., 1998; Counter et al., 1998; Lebkowski et al., 1998; Perrault et al., 2005; Piera-Velazquez et al., 2002; Sun et al., 2004; Xiaoxue et al., 2004; Yang et al., 1999), although no studies to date have been done in chondrocytes from any of the domestic animals.

Osteoarthritis is common in the dog, affecting approximately 20% of the canine population over 1 year of age (Johnston and Budsberg, 1997), and it is the progressive degeneration and loss of articular cartilage which is the most significant prognostic factor. Increasing the lifespan of chondrocytes is thus an important approach as a means of reversing articular degradation, providing cartilage grafts for joint-resurfacing or producing populations of immortal chondrocytes for further analyses. This study examined whether hTERT could be used to extend the lifespan of normal and OA canine chondrocytes without transforming the cells. In addition, we studied the effects of stable pCIneo–hTERT transfection on the expression of collagen types I and II and matrix metalloproteinase 1 (MMP-1).

Materials and methods

Chondrocyte isolation and cell culture

Slices of canine articular cartilage were aseptically removed from the femoral head of either a freshly euthanased animal or from a surgically removed femoral head following a total hip replacement procedure. The use of these tissues was approved by the Faculty of Veterinary Medicine, Ethics and Welfare Committee.

Chondrocytes were isolated using sequential enzymatic digestion by trypsin and bacterial collagenase. Suspensions were filtered through a 100 μm mesh to remove any large matrix fragments, pelleted by centrifugation and resuspended in an appropriate volume of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin/streptomycin, 0.25 μg/mL fungizone (complete DMEM). Cell viability was assessed by trypan blue exclusion and always exceeded 90%. 293T cells were purchased from ATCC.

Transfections

Plasmid DNA was introduced into chondrocyte cultures by nucleofection using the Amaxa Nucleofector in conjunction with the human chondrocyte nucleofector kit (Amaxa) according to the manufacturer's instructions. Briefly, after monolayer expansion, 1×10^6 cells were resuspended in 100 μL of nucleofector solution and 1 μg of either pCIneo or pCIneo–hTERT was added. The hTERT construct pBabehTERT was kindly provided by Professor Nicol Keith (Centre for Oncology and Applied Pharmacology, University of Glasgow) and the hTERT fragment was subcloned into pCIneo. Pooled populations of transfectants were selected by the addition of G418 (500 μg/mL) to the culture medium for 2–3 weeks. Transfected cell populations were cultured in complete DMEM containing 500 μg/mL G418. Table 1 displays the donor animals from which the chondrocytes were harvested and cultured.

Chondrocyte proliferation and population doubling assessment

Duplicate cultures of transfected chondrocytes were harvested by trypsinisation every 7–10 days and the cell numbers determined by cell counting. Population doublings (PD) were calculated using the equation: $PD = Log(N/N_0)/Log\,2$, where N_0 is the number of cells plated and N is the number of cells harvested at each passage. Cells were described as being senescent when cells became flattened, enlarged and failed to grow for a period of 4 weeks indicating a loss of proliferative capacity.

Alginate encapsulation of chondrocytes

The preparation of chondrocytes in alginate beads was performed as previously described (Guo et al., 1989). For recovery of chondrocytes, the beads were washed three times in phosphate buffered saline (PBS) and resuspended in PBS containing 50 mM EDTA until the beads had dissolved. Recovered cells were pelleted by centrifugation at 200 *g* for 10 min and stored at −80 °C.

Anchorage independence assay

Cells (5×10^5) were added to 10 mL methocel medium (0.9% methyl cellulose, 20% FCS in complete DMEM) and transferred to a bacterial grade 100 mm Petri dish. The dishes were left for 7–10 days before counting colonies >0.1 mm in diameter.

Telomerase activity assay

Cultured cells were harvested, washed twice in PBS and lysed in ice-cold CHAPS lysis buffer. After incubation at 4 °C for 30 min and a centrifugation of 16,000 *g* for 25 min at 4 °C, the supernatants were transferred to sterile diethyl pyrocarbonate (DEPC) treated tubes and were kept frozen at −80 °C until the assay was carried out. Protein concentrations were determined by bicinchoninic acid (BCA) assay (Sigma–Aldrich), according to the manufacturer's instructions.

Telomerase activity was assessed using the TRAPeze polymerase chain reaction (PCR) based telomeric repeat amplification protocol (Chemicon) according to the manufacturer's recommendations. Typically, TRAP assays were performed on 1 μg of protein extract and 20 μL of the PCR mixture was analysed on a 4–12% polyacrylamide gel, stained with ethidium bromide and visualized under UV light.

Assessment of telomere lengths

Samples were digested with proteinase K before DNA extraction by standard procedures. Telomere restriction fragment (TRF) length was measured by Southern blot hybridization following the protocol outlined in the TeloTAGGG Telomere Length Assay Kit. Kit (BD PharMingen). For a detailed description, see Nasir et al. (2001).

Western blotting

Chondrocyte protein extracts were quantified by BCA assay and 20 μg samples were loaded onto a 4–12% bis–tris SDS-polyacrylamide gel (Invitrogen). Samples were electrophoresed at 200 V for 50 min and transferred to nitrocellulose membranes. Membranes were incubated with a specific antibody against MMP-1 (Abcam) followed by a horse-radish-peroxidase-conjugated species-specific secondary antibody (Sigma–Aldrich). For loading control assessment, membranes were stripped and re-probed with a specific antibody against alpha-actin (Abcam). Immu-

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Table 1
Primary cell cultures derived from normal and OA canine donors

Name	Donor (normal)	Age (years)	Name	Donor (OA)	Age (years)
CncN1	Staffordshire terrier cross	3*	CncOA1	Golden Labrador	1
CncN2	Doberman	3*	CncOA4	Staffordshire terrier cross	1.5

Cells were subsequently transfected with pCIneo (*n*) or pCIneo–hTERT (*T*) and transfected cells populations were selected by the addition of G418 to the culture medium.
* Approximate age at post-mortem as assessed by a qualified veterinarian.

noreactive bands were detected using the ECL plus detection system (Amersham Bioscience) according to the manufacturer's instructions.

Primer design

Primer sequences for all genes examined were designed with the aid of the Primer Express software package using canine and human mRNA information published on the NCBI database. Primer products of the correct size were confirmed by non-quantitative RT-PCR and agarose gel electrophoresis. Table 2 lists the name, sequence and product size of each primer set and primer/probe combination used in this study.

RT-PCR for hTERT

Total RNA was isolated from chondrocytes using the RNAeasy (Qiagen) according to the manufacturer's instructions. cDNA was synthesised from 100–500 ng of chondrocyte RNA with the Superscript III first strand synthesis kit (Invitrogen) using the gene specific primer hTERT R (Table 2). The resulting cDNA was used as a template in subsequent PCR reactions using Platinum Taq polymerase (Invitrogen) according to the manufacturer's guidelines and the hTERT specific primers hTERT F and hTERT R (Table 2). PCR products were analysed by agarose gel electrophoresis.

Quantitative RT-PCR

Total chondrocyte RNA was prepared as above. Quantitative analysis of canine GapDH, collagen type I, collagen type II and MMP-1 gene expression was performed using 50–100 ng of total chondrocyte RNA and analysed using the Applied Biosystems 7500 Real-time PCR system (Applied Biosystems). Invitrogen Superscript III qRT-PCR was used in conjunction with the appropriate primers (Table 2) for each reaction. In these reactions, the gene-specific reverse primer was used for first strand cDNA synthesis and the subsequent PCR step. A 30 min, 50 °C denaturing step was used, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Fluorescence measurements (FAM, ROX and TAMRA) were collected every cycle at 60 °C to provide quantitative, real-time analyses.

Relative quantification (RQ) and statistical analysis

RQ calculations were performed using the efficiency corrected $2^{-\Delta\Delta Ct}$ method on Ct values from qRT-PCR experiments with the Relative Expression Software Tool (REST) 2005 package. Statistical analysis was performed as part of the RQ calculations and P values of <0.05 were considered to be statistically significant.

Results

Telomerase activity in canine chondrocytes transfected with hTERT

As shown in Fig. 1, RT-PCR confirmed expression of hTERT in both the monolayer cultured (a) and alginate cultured (b) cells stably transfected with pCIneo–hTERT. These cells were then assessed for telomerase activity. Fig. 2 shows representative polyacrylamide gels of one set of normal and one set of OA chondrocytes transfected with pCIneo–hTERT or pCIneo. Minimal telomerase activity was observed in the early passage normal chondrocytes transfected with pCIneo, (Fig. 2a, lane 1); however, the same cells transfected with pCIneo–hTERT exhibit telomerase activity at early (6 PDs) and late passage (28 PDs) (Fig. 2a, lanes 3 and 5). In the OA chondrocytes, the cells transfected with pCIneo show little or no telomerase

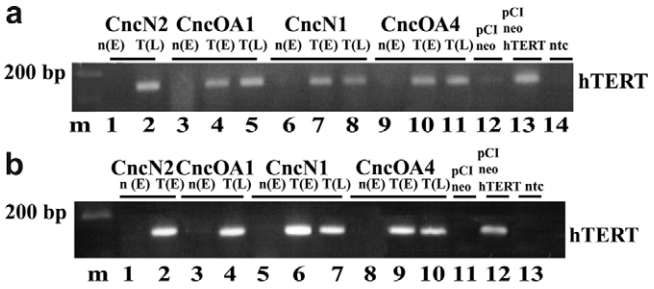

Fig. 1. Detection of hTERT mRNA expression with RT-PCR. Total RNA from monolayer cultured (a) or alginate embedded (b) canine chondrocytes stably transfected with either pCIneo (*n*) or pCIneo–hTERT (*T*) was analysed (*E*/*L* denotes early or late passage). All hTERT transfected cultures produced a band of approximately 154 bp whereas all cultures transfected with the control vector did not. Control PCR reactions employing the plasmids pCIneo and pCIneo–hTERT confirmed the specificity of the primers used. NTC = no template control.

Table 2
Primers and probes used in RT-PCR and qRT-PCR

Gene	Forward primer 5′-3′	Reverse primer 5′-3′	Probe 5′-3′	Amplicon size
hTERT	ggcaggacgcgtggaccgagtgaccgt	gatgtggatgggggcccgcgtggtgct	NA	154 bp
Canine collagen I	gccgcttcacctacagtgtca	gaggtcttggtggttttgtattcg	tacgacggctgcagcagtcacactg	93 bp
Canine collagen II	cagcaggttcacatatactgttctga	cgatcatagtcttgccccactt	tggctgcacgaaacacaccgg	73 bp
|Canine MMP-1	ccgtgacaattctcccttttgat	cctagatttgggcctggtga	acccaatggaaacctcgctcatgctt	72 bp
Canine GapDH	ccccacccccaatgtatca	gtcgtcatatttggcagctttct	tgtggatctgacctgccgcctg	65 bp

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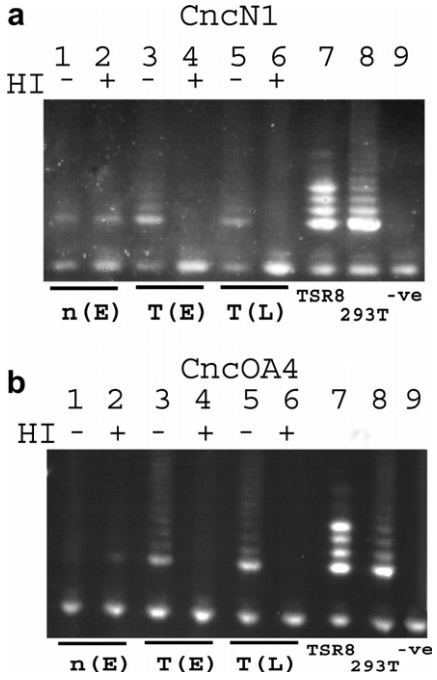

Fig. 2. Telomerase activity in stably transfected chondrocytes. CHAPS extracts from monolayer cultured normal (CncN1) or OA (CncOA4) chondrocyte cell populations stably transfected with either pCIneo (*n*) or pCIneo–hTERT (*T*) were assayed for telomerase activity (*E*/*L* denotes early or late passage) by the telomeric repeat amplification protocol (TRAP). (a) Lanes 1–6 show the telomerase activity of normal transfected chondrocytes ± heat inactivated controls. (b) Lanes 1–6 show the telomerase activity of OA transfected chondrocytes ± heat inactivated controls. TSR8 and 293T represent positive controls supplied with the assay kit. Negative = CHAPS lysis buffer only.

activity (Fig. 2b, lane 1) whereas the hTERT expressing cells show telomerase activity at early (6 PDs) and late 23 PDs) passage (Fig. 2b, lanes 3 and 5, respectively).

Telomerase activity extends the replicative lifespan of monolayer cultured chondrocytes

The results (Fig. 3a and b) show that the normal chondrocytes CncN1n and CncN2n, transfected with pCIneo, stopped proliferating after 40–50 days in culture, reaching 7 and 4 population doublings, respectively. In contrast, the CncN1T and CncN2T cells, transfected with pCIneo–hTERT, show little indication of becoming senescent. So far, the CncN1T and CncN2T cells have reached 28 and 17 population doublings respectively. Similar results were obtained with chondrocytes derived from OA donors (Fig. 3c and d). The OA chondrocytes CncOA1n and CncOA4n entered senescence after approximately 50 days and 40 days, respectively, for a maximum of seven population doublings. The CncOA1T and CncOA4T cells continue to grow, reaching 23 and 19 population doublings to date respectively. hTERT had no major effect on the growth kinetics, suggesting that exogenous telomerase expression does not increase the rate of chondrocyte proliferation.

Telomerase activity maintains chondrocyte telomere length in monolayer culture

As shown in Table 3, TRF analyses show that freshly isolated chondrocytes from a normal donor (CncN1) exhibit a mean telomere length of 19.07 kb. After stable transfection with pCIneo, subsequent selection in G418, and several passages (6 PDs) the observed telomere length is shortened to 17.65 kb. In contrast, cells expressing hTERT show stabilisation of the telomere lengths at both early (6 PDs) and late (28 PDs) exhibiting mean telomere lengths of 18.49 and 18.54 kb, respectively. Similar results were

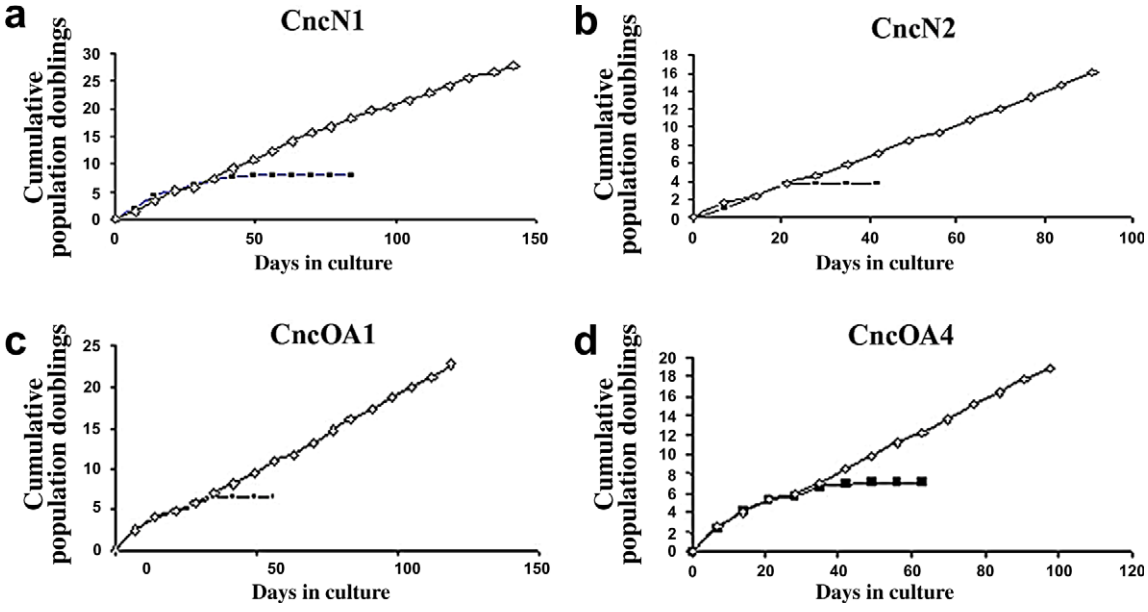

Fig. 3. Growth curves for normal (a and b) and OA (c and d) chondrocytes stably transfected with pCIneo (■) or pCIneo–hTERT (◇). Transfected chondrocyte cultures were grown in monolayer culture, trypsinised, counted and re-plated at an appropriate density. Population doublings per passage were calculated using the equation, $PD = \mathrm{Log}(N/N_0)/\mathrm{Log}2$.

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Table 3
Chondrocyte telomere length assessment by TRF analyses

Cells	Mean TRF length (kb)
CncN1	19.07
CncN1neo	17.65
CncN1TERT early	18.49
CncN1TERT late	18.54
CncOA1	15.84
CncOA1neo	11.65
CncOA1TERT early	14.54
CncOA1TERT late	15.79

Data represents mean telomere length from two genomic DNA digests from independent cultures.

observed using chondrocytes from an OA donor (CncOA1). Untransfected CncOA1 chondrocytes have a mean telomere length of 15.84 kb. Cell populations stably transfected with pCIneo showed a dramatic reduction in telomere length down to 11.65 kb after only six population doublings post-selection whereas cells stably transfected with pCIneo–hTERT exhibit telomere lengths of 14.54 kb and 15.79 kb at 6 and 23 PDs respectively. These results demonstrate that telomere lengths in both normal and OA canine chondrocytes can be stabilised by the exogenous expression of hTERT.

hTERT does not transform primary canine chondrocytes

The cells lines CncN1n, CncN1T, CncN2n, CncN2T, CncOA1n, CncOA1T, CncOA4n and CncOA4T were assayed for anchorage independent growth after approximately six population doublings. The chondrocytes expressing hTERT and the negative control cells failed to form foci, whereas the 293T positive control cells readily formed cellular aggregates indicative of tumorigenic foci (561 ± 72) (Table 4). The cells were also grown in DMEM containing low (0.1%) serum and, after 14 days incubation, nearly all the hTERT transfected and control cells had undergone cell death. In contrast, the control 293T cells exhibited a small degree of growth (data not shown).

Table 4
Anchorage independent growth

	Seeded cells	Number of colonies
Normal cell lines		
CncN1neo	5×10^5	0
CncN1TERT	5×10^5	0
CncN2neo	5×10^5	0
CncN2TERT	5×10^5	0
CncN3neo	5×10^5	0
CncN3TERT	5×10^5	0
OA cell lines		
CncOA1neo	5×10^5	0
CncOA1TERT	5×10^5	0
CncOA2neo	5×10^5	0
CncOA2TERT	5×10^5	0
CncOA4neo	5×10^5	0
CncOA4TERT	5×10^5	0
Control cell lines		
293T	5×10^5	561 ± 72
CMT-7	5×10^5	462 ± 89

Normal or OA chondrocytes were seeded into methylcellulose containing growth medium and incubated at 37 °C for 7–10 days. Colonies larger than 0.1 mm in diameter were counted under light microscopy.

Exogenous hTERT alters the expression of MMP-1 but not collagen type I or II in OA chondrocytes

The expression of collagen type I, collagen type II and MMP-1 mRNAs were assessed by qRT-PCR in both normal and OA alginate embedded chondrocytes transfected with either pCIneo–hTERT or the control vector pCIneo. Expression values were normalised to the housekeeping gene GapDH and the data are presented as changes in mRNA expression in hTERT expressing cells relative to the control cells. The results show that in normal chondrocytes expressing hTERT (Fig. 4a), there are modest reductions in the expression of collagen type I, type II and MMP-1 compared to the control cells, although the

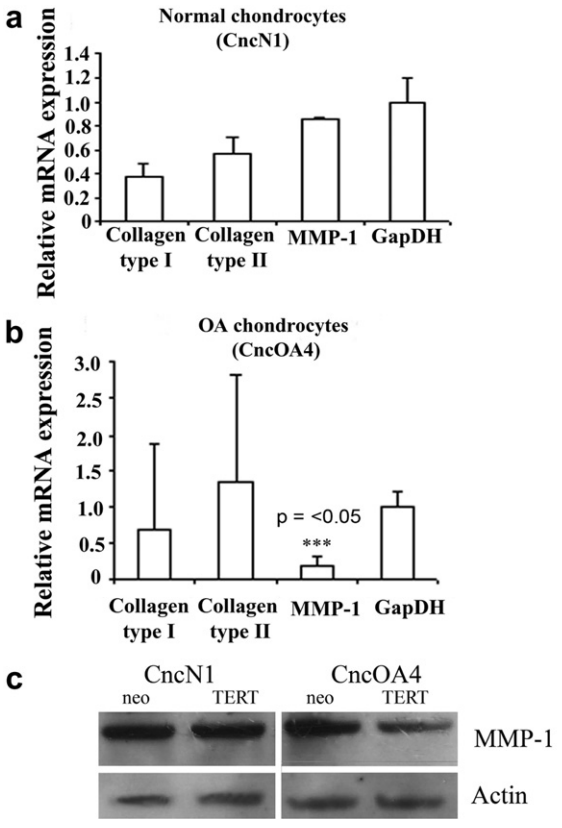

Fig. 4. qRT-PCR analysis of collagen type I, collagen type II and MMP-1 mRNA in TERT expressing chondrocytes. (a) mRNA expression in TERT expressing normal chondrocytes compared to pCIneo transfected control cells. (b) mRNA expression in TERT expressing OA chondrocytes compared to pCIneo transfected control cells. mRNA expression was normalised to the housekeeping gene GapDH. All samples were performed in triplicate and the results shown represent data from three experiments. (c) Western blot analysis of MMP-1 protein expression in transfected cell populations from one normal (CncN1) and one OA (CncOA4) donor. Alpha-actin served as a loading control.

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changes are not statistically significant ($P = 0.082$, 0.254 and 0.14, respectively).

In OA chondrocytes (Fig. 4b), hTERT expressing cells show a minor reduction in collagen type I mRNA and a slight increase in collagen type II mRNA expression as compared to the control cells. As was observed in the normal chondrocytes, the changes in the expression of the two collagens did not achieve statistical significance ($P = 0.879$ and 0.873, respectively). In contrast to the results from the normal cells however, a statistically significant change in MMP-1 expression was observed in hTERT expressing OA cells compared to the controls. The relative amounts of MMP-1 mRNA in the hTERT transfected OA cells was reduced approximately 6-fold ($P = 0.047$). Western blot analysis of normal and OA chondrocytes expressing revealed a concomitant reduction in MMP-1 protein expression in OA cells expressing hTERT compared to the control cells (Fig. 4c, lanes 3 and 4). No reduction in MMP-1 expression was observed in normal hTERT expressing chondrocytes compared to the controls (Fig. 4c, lanes 1 and 2).

Discussion

In all the cell types presently examined to date, the exogenous expression of hTERT increased the replicative lifespan and stabilised telomere lengths compared to the negative control cells chondrocytes (Bodnar et al., 1998; Counter et al., 1998; Lebkowski et al., 1998; Perrault et al., 2005; Piera-Velazquez et al., 2002; Sun et al., 2004; Veitonmaki et al., 2003; Wang et al., 2005; Xiaoxue et al., 2004; Yang et al., 1999). Our results are in agreement with these studies as we have demonstrated an increase in the proliferative potential of hTERT expressing chondrocytes compared to the controls and the stabilisation of the telomere lengths. In this study, we show that hTERT is sufficient to reactivate telomerase in canine cells. It remains to be determined whether canine TERT is able to work better than hTERT. Both the clinically affected and normal dogs were relatively young, not the age group most often associated with OA. Both clinical cases had severe OA but it is true that the age of the chondrocytes could affect their response and thus these studies need to be repeated using older donors.

The results from the in vitro transformation assays and the observation that hTERT expression has little or no effect on chondrocyte proliferation rates strongly suggest that the cells have not undergone neoplastic transformation. However, there are reports that hTERT can induce karyotype instability and premalignant phenotypes in human fibroblasts. One study showed that chromosomes of cells cultured from aged donors (102 years of age) were highly susceptible to trisomies, deletions and translocations (Mondello et al., 2003). Continued culture of these cells resulted in the ability to grow in anchorage independent conditions and form tumours in nude mice (Zongaro et al., 2005). Whether or not this genomic instability is directly due to telomerase or is associated with the donor age is still unclear. Similarly, another study showed that telomerase immortalized fibroblasts began to express the c-myc and Bmi-1 oncogenes after approximately 150 PDs, indicative of a potentially malignant alteration (Milyavsky et al., 2003).

Our data also show that the normal chondrocytes achieved senescence with considerably longer telomeres than the OA cells (17.65 kb vs. 11.65 kb respectively). Two possible explanations for this are that: (1) monolayer tissue culture accelerates the loss of telomeric repeats from specific chromosomes, and that the rate of loss from any possible senescence-associated chromosome is masked by measuring mean telomere lengths within a cell population or: (2) telomere erosion is not the only step required for the onset of premature senescence within cultured cell populations.

The dramatic loss of telomeric sequences observed in the CncOA1 donor cells reflects a rate of telomere erosion in excess of double that observed for CncN1 donor cells. As the cells were transfected and then selected in antibiotic, it is possible that the OA cells were less permissive to transfection than normal, healthy cells. This, in turn, would produce a smaller number of surviving cells which, in the case of the pCIneo transfected cells, would require them to undergo more cell divisions to achieve the same number of post-selection cells. In addition, it is possible that OA cells are more prone to oxidative stress in culture than normal cells and that the culture conditions employed accelerated the telomere loss in these cells.

Although not statistically significant, the slight increase in expression of collagen type II mRNA in hTERT expressing OA chondrocytes is encouraging, suggesting a positive anabolic effect. The observation that MMP-1 mRNA and protein expression are down-regulated in hTERT expressing chondrocytes is surprising since increased MMP-1 expression is frequently associated with cells with an active telomerase holoenzyme such as tumour cells. Whether this is a direct effect of hTERT on MMP-1 or the result of downstream signalling events remains unclear at this time. Since MMP-1 is a known degradative factor involved in cartilage destruction in OA, any down-regulation of this protein will help to slow the progression of the disease.

Conclusions

The data presented in this study suggest that a telomerase based gene therapy treatment has the potential to be a safe and novel approach to overcoming chondrocyte senescence and promoting cartilage repair. Such an approach will prolong the normal cellular activity of transfected chondrocytes and prevent the degradative matrix changes that are associated with senescence.

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