Hypermethylation of the hTERT promoter inhibits the expression of telomerase activity in normal oral fibroblasts and senescent normal oral keratinocytes

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Telomerase activity in human cells closely correlates with the expression of its catalytic subunit, telomerase reverse transcriptase (hTERT). Previously, we reported the lack of telomerase activity in normal human oral fibroblasts (NHOF) and the diminution of telomerase activity during senescence in normal human oral keratinocytes (NHOK). To investigate the underlying mechanisms of telomerase regulation in both cell types, we analysed the expression, promoter activity, and methylation status of the hTERT gene. The expression of hTERT mRNA diminished in senescent NHOK, but was not detected in NHOF at any stage of replication. An exogenous hTERT promoter was active in NHOF and in senescent NHOK, indicating that the lack of hTERT gene expression resulted from alteration of the endogenous hTERT promoter. Since methylation is involved in the silencing of numerous genes, we carried out DNA methylation assays. The assay revealed that the hTERT promoter was hypermethylated in NHOF and was gradually methylated during senescence in NHOK. Treatment of NHOF and senescent NHOK with the demethylating agent 5-aza-2′-deoxycytidine restored the expression of endogenous hTERT mRNA. Our results suggest that hypermethylation of the hTERT promoter plays a critical role in the negative regulation of telomerase activity in normal human oral cells.

Keywords: hTERT; hypermethylation; senescence; fibroblasts; keratinocytes

MATERIALS AND METHODS

Cells and culture conditions

Primary cultures of NHOF were established from explants of the gingival connective tissue excised from patients undergoing oral
surgery. The cells that proliferated outwardly from the explant culture were continuously cultured in 100-mm culture dishes, in DMEM/medium 199 (4:1) containing foetal bovine serum (Gemini Bioproducts, Calabasas, CA, USA), and gentamicin (50 μg ml⁻¹). Primary NHOK were prepared from separated epithelial tissue, and serially subcultured in keratinocyte growth medium (KGM, Clonetics, Charlotte, NC, USA) containing 0.15 mM Ca²⁺ and serially subcultured in keratinocyte growth medium (KGM, Clonetics, Charlotte, NC, USA) containing 0.15 mM Ca²⁺, as described previously (Kang et al, 1998). Adenovirus transformed human embryonic kidney cells 293, obtained from the American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM/Ham's F-12, containing 10% foetal bovine serum (Gemini Bioproducts) and gentamicin (50 μg ml⁻¹). SCC-4 (human squamous cell carcinoma cell line) was obtained from American Type Culture Collection (Manassas, VA, USA), and cultured in DMEM/Ham's F-12 containing 10% foetal bovine serum (Gemini Bioproducts) and gentamicin (50 μg ml⁻¹).

Cell population doublings (PD) were determined after sub-culturing to postmitotic stage. At the end of each passage, PD was calculated by the formulation, \( 2^{\frac{C_f - C_i}{N}} = (Ci/Cf) \), where \( N \) denotes PD, \( C_f \) the total cell number harvested at the end of a passage, and \( C_i \) the total cell number of attached cells at seeding. The PD time was calculated by dividing the duration of culture in hours by the number of PD.

**Analysis of telomerase activity**

Cellular extracts were prepared by using CHAPS (lysis buffer) provided from the TRAP-eze Telomerase Detection Kit (Intergen Norcross, GA, USA), as recommended by the manufacturer. Telomerase activity was determined using the TRAP-eze Telomerase Detection Kit as described previously (Kang et al, 1998). Each TRAP reaction contained a cellular extract equivalent to 1 μg protein. The PCR products were electrophoresed in 12.5% non-denaturing polyacrylamide gels, and the radioactive signals were detected by Phosphorimage (Molecular Dynamix, Sunnyvale, CA, USA).

**Analysis of endogenous hTERT expression**

Total RNA was isolated from the cells using Trizol™ reagent (Invitrogen Carlsbad, CA, USA) and further purified through RNAeasy columns (Qiagen, Chatsworth, CA, USA). Following isolated RNA solution in 7.5 μl H₂O, the reverse transcription (RT) reaction was performed in first-strand buffer (Invitrogen) containing 200 U SuperScript II (Invitrogen), 40 U RNase inhibitor (Perkin-Elmer, Foster City, CA, USA), 10 μM dithiothreitol, 250 ng random hexamer (Perkin-Elmer), and 2.5 μM dNTP. The annealing reaction was carried out for 10 min at 25°C, and cDNA synthesis was performed for 50 min at 42°C, followed by 10 min incubation at 70°C for enzyme inactivation.

To amplify hTERT cDNA, PCR reaction was performed with 1 μl RT product using the primers 5'-GCTGTAGCTGTACTCTTGGC-3' (forward) and 5'-GGCCACAGCCTGTTTCCATGTG-3' (reverse). The primer set amplified hTERT mRNA species encoding functional full-length (457 bp) or defective spliced isoforms (421, 275, or 239 bp) (Ding et al, 2002). PCR products were separated in 2% agarose gels. The hTERT signals were further amplified by Southern hybridisation using 32P-[dCTP]-radiolabelled probe synthesised from hTERT cDNA, as described in detail previously (Kang et al, 2002).

**Analysis of hTERT promoter activity**

A pGL3-TRTP containing a 1670 bp fragment (−1665 to 5) of full-length hTERT promoter upstream of the firefly luciferase gene in the pGL3-basic (Promega Madison, WI, USA), kindly provided by Dr J Carl Barrett (National Institute of Environmental Health Science), was used for the hTERT promoter assay. Prior to transfection, a six-well plate with approximately 5 x 10⁶ cells per well was inoculated and cultured for 24 h. The pGL3-basic (2 μg well⁻¹) or the pGL3B-TRTP vectors (2 μg well⁻¹) were transfected using Lipofectin reagent (BRL Life Technologies, Inc., Gaithersberg, MD, USA). For better comparison among cells with different transfection efficiencies, the pGL3-control plasmid (2 μg well⁻¹; Promega), which has the firefly luciferase gene under the transcriptional control of SV40 enhancer/promoter, was also transfected into each cell and used for normalisation of the activities shown by the pGL3-B-TRTP construct. Cells were collected 48 h after transfection and cell lysates prepared according to Promega’s instruction manual. Luciferase activity was measured using a luminometer (Promega).

**Methylation analysis of hTERT promoter**

Methylation of the hTERT promoter was studied using methylation-specific PCR with bisulphite-modified DNA and 5-aza-2'-deoxycytidine (5-aza-CdR) treatment (Dessain et al, 2000). For methylation-specific PCR with bisulphite-modified DNA, genomic DNAs from cells were isolated using Qiagen Tissue/Blood Kit (Qiagen), and bisulphite-modified according to published methods (Dessain et al, 2000). Promoter regions of hTERT were amplified from the nonmodified and the modified DNA. PCR reaction products were electrophoresed in 2% agarose and amplified DNA fragments were directly visualised under UV illumination.

Cells were incubated in the culture medium with 10 μM 5-aza-CdR for 24 h on days 3 and 5, and then harvested on day 7 for analysis. Total RNA was prepared using Trizol™ reagent (Invitrogen). Randomly primed cDNAs were reverse-transcribed and hTERT cDNA was amplified as described above.

**RESULTS**

**Correlation between the expression of endogenous hTERT and telomerase activity in NHOF and NHOK**

Our previous studies have shown the absence of telomerase activity in NHOK, its presence in exponentially replicating NHOK, and its gradual diminution during senescence in NHOK (Kang et al, 1998). This finding was confirmed in the present investigation, subjecting the exponentially replicating NHOF and NHOK, and senescent NHOK, to the telomeric repeat amplification (TRAP) assay (Figure 1A). Telomerase activity was not detected in replicating NHOF or in senescing NHOK, but was detected in exponentially proliferating NHOK (Figure 1).

The regulation of telomerase activity by expression of the hTERT gene was confirmed. The expression of full-length and splicing variants of hTERT was also determined using primers flanking the RT domain of hTERT (Ding et al, 2002). The full-length hTERT mRNA containing the functional RT domain was detected in exponentially replicating NHOK as a 457 bp band (Figure 2). The spliced variant of hTERT mRNA, also known as the β-deletion transcript containing a nonfunctional RT domain, was also identified in exponentially replicating NHOK as a 275 bp band (Figure 2). The β-deletion transcript causes a 183 bp deletion (bases 2342–2524) resulting in nonsense mutation, truncating the hTERT protein before the conserved RT motifs (Ulancer et al, 1998). hTERT mRNA was not detected in replicating NHOK or senescent NHOK which lacked telomerase activity (Figure 2). The presence of hTR mRNA in all tested cells indicated that hTR was not the limiting factor of the telomerase activity in NHOF and senescent NHOK (Figure 2).

**Transcriptional activity of an exogenous hTERT promoter in NHOF and NHOK**

The absence of hTERT mRNA in NHOF and senescent NHOK could result from either one of the following forms of negative
control: (1) repressive alteration in the cis-acting hTERT promoter region or (2) defect in trans-acting regulatory factors required for promoter activity. To distinguish between the two possibilities, we examined the transcriptional activity of an exogenous hTERT promoter in NHOF and NHOK. SCC-4, a telomerase-positive human oral cancer cell line, served as positive control (Kang et al., 1998). The pGL3B-TRTP luciferase reporter plasmid containing a full-length hTERT promoter (nucleotides −1665 to +5) was transiently transfected into cells. The luciferase activity of pGL3B-TRTP was measured and compared with that of the pGL3-control plasmid, which was driven by the SV40 enhancer/promoter and used to monitor the transfection efficiency (Horikawa et al., 1999; Takakura et al., 1999).

As shown in Table 1, the pGL3B-TRTP plasmid expressed luciferase activity, that is, 9.8% of the pGL3-control, in the telomerase-positive human oral cancer cell line SCC-4. The same level of activity was found in exponentially replicating (PD 10) and senescing (PD 20) NHOK (7.0 and 6.9%, respectively). A similar level of activity (10.9% of the pGL3-control) was detected in hTERT-negative NHOF. These findings indicate that the necessary factors for hTERT expression were present and active in NHOF and senescent NHOK. It also suggests that negative alteration of the hTERT promoter is involved in repression of endogenous hTERT expression.

### Methylation of the hTERT promoter in NHOF and senescent NHOK

Previous studies have shown that (1) promoter hypermethylation is frequently associated with gene silencing (Herman et al., 1999), (2) the telomerase gene has a typical CpG island domain for methylation in its promoter (Horikawa et al., 1999; Takakura et al., 1999), and (3) the human hTERT gene promoter is hypermethylated in cells with reduced telomerase activity (Devereux et al., 1999; Bechter et al., 2002). These findings suggested that the absence of hTERT gene expression in NHOF and senescent NHOK might be due to hypermethylation of the endogenous hTERT promoter. In the hTERT promoter, two CpG islands, the transcription start site and regions 3′ to the translation start site, have been well characterised (Conig et al., 1999; Horikawa et al., 1999; Takakura et al., 1999; Dessain et al., 2000). Therefore, it was possible to determine the presence of methylation in both CpG islands in NHOF and senescent NHOK by using bisulphite treatment of DNA and subsequent methylation-specific PCR (Dessain et al., 2000).

In NHOF, as shown in Figure 3, the region 3′ to the translation start site (TL) was completely methylated, while the region 3′ to the transcription start site (TC) was not methylated (Figure 3A). In rapidly proliferating NHOK (PD 11) that expressed hTERT mRNA and telomerase activity, both CpG islands in the hTERT promoter were hypomethylated (Figure 3B). Senescing NHOK (PD 20) showed methylation in both CpG islands (Figure 3C). With a reduced number of PCR cycles (15 cycles), the fraction of cells containing the methylated promoter was greater than that of cells containing the unmethylated promoter (Figure 3C). The methylation status of the hTERT promoter in the telomerase-positive cell line SCC-4 was also examined. Both CpG islands in the hTERT promoter were not methylated in the cell line (Figure 3D).

### Introduction of endogenous hTERT expression in NHOF and senescing NHOK exposed to 5-aza-CdR

We investigated whether hypermethylation was solely responsible for repression of the hTERT gene expression by exposing the cells to 5-aza-CdR, the potent demethylating agent (Bender et al., 1998). The concentrations of 5-aza-CdR utilised in the present study were based on the studies reported by others (Devereux et al., 1999;
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hTERT methylation in normal human oral cells

Table 1 Exogenous hTERT promoter activity in NHOK, NHOF, and senescent NHOK

| Cells          | Telomerase activity | pGL3-basic (no promoter) | pGL3B-TRTP (hTERT promoter) | pGL3-control (SV40 promoter) |
|---------------|---------------------|--------------------------|-----------------------------|------------------------------|
| SCC-4         | +                   | 1.7 ± 0.5 (0.03%)        | 610 ± 61 (9.8%)             | 624 ± 825 (100%)            |
| NHOK (PD 10)  | +                   | 0.01 ± 0.02 (0%)         | 244 ± 13 (7%)              | 3507 ± 113 (100%)          |
| NHOK (PD 20)  | −                   | 0.004 ± 0.002 (0%)      | 607.7 ± 23 (69 %)          | 878 ± 32 (100%)            |
| NHOK (PD 24)  | −                   | 0.01 ± 0.02 (0%)        | 115.4 ± 4 (109%)           | 1059 ± 148 (100%)          |

*Mean luciferase activity and standard deviation. The activity of pGL3-basic or pGL3B-TRTP was normalised with that of pGL3-control. *SCC-4, telomerase-positive human squamous cell carcinoma cell line. Percentage of pGL3-control.

Dessain et al, 2000). Furthermore, we determined the cytotoxic effect of 5-aza-CdR (at a range of 1–10 μM) in exponentially proliferating NHOF and NHOK for up to 3, 7, or 10 days, and found that 1 or 5 μM of 5-aza-CdR did not demonstrate any cytotoxic effect in these cells during the 7-day period, by determining the proliferation rate of cells as described elsewhere (Kang et al, 2000). NHOF and senescing NHOK were cultured in the absence or presence of 5 μM 5-aza-CdR for 3, 5, and 7 days, and

DISCUSSION

Activation of telomerase activity is thought to be a critical step in cellular immortalisation and transformation. Since telomerase activity is primarily regulated through hTERT expression, understanding of hTERT regulation in normal cells is crucial for the understanding of carcinogenesis. Our present studies provide evidence that methylation of the hTERT promoter is responsible

British Journal of Cancer (2003) 89(8), 1473 – 1478 © 2003 Cancer Research UK
promoter activity was not activated or inactivated during
and (2) the transcriptional machinery necessary for the
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hTERT/C0 included the 59 bp region (nt NHOK, we found significant transcriptional activity in all tested
expression of the inactive
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5-aza-CdR treatment is not
- deletion splice variant is longer than the full-
hTERT mRNA, and autosecretion of TGF-
immortalised endocervical cells (Ding et al, 2002). According to a previous report (Yi et al, 2001), a β-deletion splice variant was dominantly expressed (80 – 90% of total hTERT mRNA) in various human epithelial cells. Since NHOK is an epithelial cell, the domination of the β-deletion splice variant is not surprising. Although the reason for the dominant reactivation of the β-deletion splice variant upon the 5-aza-CdR treatment is not known, we could not rule out the possibility that the half-life (stability) of the β-deletion splice variant is longer than the full-length hTERT mRNA, and autosecretion of TGF-β retains high expression of the inactive β-deletion splice variant in NHOK culture (Cerezo et al, 2002).

After testing the transcriptional activity of an exogenous hTERT promoter in NHOF, rapidly proliferating NHOK and senescent NHOK, we found significant transcriptional activity in all tested cells, regardless of the presence of telomerase activity. The promoter region (nt – 1665 to +5) examined in this study included the 59 bp region (nt – 208 to –150) that was responsible for the maximal promoter activity (Horikawa et al, 1999). The data showed that in NHOF and senescing NHOK: (1) the transcriptional machinery necessary for the hTERT promoter activity in replicating NHOF functioned as well as in NHOK-expressing hTERT mRNA and (2) the transcriptional machinery necessary for hTERT promoter activity was not activated or inactivated during

for the repression of hTERT expression, which results in the lack of telomerase activity in NHOF and senescent NHOK.

Transcriptional activation has been considered a major mechanism for the regulation of the hTERT gene (Poole et al, 2001). Consistent with previous observations (Kim et al, 1994; Shay and Bacchetti, 1997), we found that expression of hTERT is a limiting factor for telomerase activity in NHOF and NHOK. Also, the presence of hTERT mRNA encoding functional or defective spliced isoforms has been demonstrated in several types of normal and pathogenic human cells (Ulaner et al, 1998, 2000; Liu et al, 1999). The splicing pattern of hTERT mRNA has been considered part of a regulatory mechanism for telomerase activity, but the details are unknown (Kilian et al, 1997; Ulaner et al, 1998, 2000; Wick et al, 1999). The β-deletion transcript causes premature termination of translation, resulting in truncation of the protein, and has been observed in various cell types (Nakamura et al, 1997; Wick et al, 1999; Ding et al, 2002). In the present study, we have detected the full-length and the β-deletion transcripts of hTERT in normal oral keratinocytes. Similar to our observations, coexpression of the full-length and the β-deletion transcripts have been reported in normal ovarian tissues (Ulaner et al, 2000) and human papillomavirus-immortalised endocervical cells (Ding et al, 2002). According to a previous report (Yi et al, 2001), a β-deletion splice variant was dominantly expressed (80 – 90% of total hTERT mRNA) in various human epithelial cells. Since NHOK is an epithelial cell, the domination of the β-deletion splice variant is not surprising. Although the reason for the dominant reactivation of the β-deletion splice variant upon the 5-aza-CdR treatment is not known, we could not rule out the possibility that the half-life (stability) of the β-deletion splice variant is longer than the full-length hTERT mRNA, and autosecretion of TGF-β retains high expression of the inactive β-deletion splice variant in NHOK culture (Cerezo et al, 2002).

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senescence in NHOK. Therefore, the absence of hTERT expression does not appear to be caused by a defective transcriptional machinery, but by an altered endogenous hTERT promoter.

In contrast with other reports of unmethylated hTERT promoters in normal fibroblasts (Devereux et al, 1999; Dessain et al, 2000), our studies show that hTERT expression appears to be correlated with the methylation status of the promoter in NHOF, suggesting that promoter methylation does provide a means to regulate hTERT expression in cells. To rule out the possibility of experimental errors and donor variation, we performed two assays in three strains of NHOF from different donors and obtained consistent reproducible results. Therefore, the hTERT promoter is tissue-specifically methylated and silenced in normal human fibroblasts (Imamura et al, 2001; Shiota et al, 2002).

Previously, we reported that replication arrest preceded differentiation in NHOK serially subcultured in vitro (Kang et al, 2000). The silencing of hTERT during squamous cell differentiation was demonstrated (Harle-Bachor and Boukamp, 1996). Based on these observations, we speculate that the silencing of hTERT due to promoter methylation is also in part associated with the silencing of hTERT during squamous differentiation in vivo. Hypermethylation of the hTERT promoter during senescence in normal oral keratinocytes was correlated with the diminution of telomerase activity and hTERT mRNA expression. The involvement of promoter methylation in hTERT gene regulation during senescence in oral keratinocytes was further confirmed by induction of hTERT expression in senescent NHOK exposed to 5-aza-CdR. This finding supported the concept that the differentiation of cells is associated with changing methylation status at many loci in the genome (Shiota et al, 2002). While hTERT expression was restored by exposure to 5-aza-CdR, telomerase activity was not. Although the reason for inability of the chemical to restore telomerase activity is not known, we speculate that the inhibitory effect of 5-aza-CdR on DNA synthesis and telomerase activity may be the cause. 5-aza-CdR inhibited both DNA synthesis and telomerase activity in exponentially replicating NHOK (our unpublished data).

ACKNOWLEDGEMENTS

We thank Dr JC Barrett (NIEHS) for providing the pG3B-TRTP plasmids. This work was supported in part by the grants (DE14147 and DE14635) funded by the National Institute of Dental and Craniofacial Research (NIDCR).

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