Molecular Characterization of Human Telomerase Reverse Transcriptase-immortalized Human Fibroblasts by Gene Expression Profiling: Activation of the Epiregulin Gene\textsuperscript{1,2}

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Abstract

Reconstitution of telomerase activity by ectopic expression of telomerase reverse transcriptase (hTERT) results in an immortal phenotype in various types of normal human cells, including fibroblasts. Despite lack of transformation characteristics, it is unclear whether hTERT-immortalized cells are physiologically and biochemically the same as their normal counterparts. Here, we compared the gene expression profiles of normal and hTERT-immortalized fibroblasts by using a cDNA microarray containing 20,736 cDNA clones and identified 172 dysregulated genes or expressed sequence tags (ESTs). One of the highly expressed genes in the hTERT-immortalized fibroblasts (hTERT-BJ cells) encodes epiregulin, a potent growth factor. Blockade of epiregulin reduced the growth of hTERT-BJ cells and colony formation of hTERT-transformed fibroblasts. Moreover, inhibition of epiregulin function in immortal hTERT-BJ cells triggered a senescence program. Our results suggest that both activation of telomerase and subsequent induction of epiregulin are required for sustained cell proliferation. Given the significant difference in gene expression profiles between normal and hTERT-immortalized fibroblasts and the close relationship between epiregulin and tumorigenesis, we conclude that hTERT-immortalized cells may not replace their normal counterparts for studies of normal cell biology and that the use of hTERT for expansion of normal human cells for therapeutic purposes must be approached with caution.

Introduction

Normal human somatic cells have a finite replicative life span. For instance, cultured fibroblasts are only capable of dividing 50–60 times before entering senescence, which is characterized by an irreversible growth arrest (1). Convincing evidence shows that the timing of cellular senescence in humans is predominantly dictated by the length of telomeres, repetitive TTAGGG sequences at the ends of each chromosome (1, 2). Telomere sequences are synthesized by telomerase, an RNA-dependent DNA polymerase that contains two critical components, the RNA template and the catalytic unit or hTERT\textsuperscript{5} and a number of other elements (3). Most somatic cells lack telomerase activity and progressively lose their telomeric DNA with each division because of the end replication problem and other cellular/molecular events (1). Extremely shortened telomeres trigger a senescence program and thereby impose a limit to cellular life span. Consistent with this hypothesis, reconstitution of telomerase activity by forced expression of hTERT cDNA has been shown to enable human fibroblasts and other cell types to stabilize their telomere sizes and to acquire immortality (2).

Given the observations that hTERT or telomerase-immortalized fibroblasts lack transformation characteristics nor exhibit genomic instability, it has been proposed that indefinite expansion of normal human cells by hTERT might be useful for biochemical and physiological studies of normal cell growth and differentiation, and for cell-based therapies and genetic manipulations, etc (2, 4, 5). However, Stewart et al. have recently found that hTERT is capable of contributing to malignant transformation of human fibroblasts by a telomere length-independent mechanisms (6). In addition, another recent study showed that the oncogene c-Myc was activated in hTERT-immortalized human epithelial cells, suggesting a potential transformation risk (7). Given the possible future applications of these cells, it will be important to clarify any biological differences at molecular levels between normal and hTERT-immortalized cells and to determine whether hTERT-immortalized cells have potentials to develop into malignant cells. Because many cellular processes are regulated at gene expression levels, we investigated the expression profile of normal and hTERT-immortalized fibroblasts by using high density DNA microarray. Our results show that the transcription profile is considerably different in hTERT-BJ cells compared with normal mortal fibroblasts. We additionally observed that epiregulin, a member of the EGF family (8), is highly up-regulated in hTERT-BJ cells and plays a critical role in the maintenance of sustained proliferation of these cells.

Materials and Methods

Cells, Cell Culture, and Cell Proliferation Assay. Normal human foreskin fibroblasts, hTERT-BJ cells derived from foreskin fibroblasts (Clontech; Ref. 2), and hTERT/SV40/H-ras-transformed BJ cells (kindly provided by Dr. Robert A. Weinberg; Ref. 9) were maintained in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% of FCS, 100 units/ml penicillin, and 2 mM l-glutamine. Normal human embryonic lung fibroblasts LFI cells and their hTERT-expressing counterparts at early and late passages LFI-hTERTp8 and LFI-hTERTp22 were previously described (10).

\textsuperscript{5} The abbreviations used are: hTERT, human telomerase reverse transcriptase; hTERT-BJ, hTERT-immortalized skin fibroblasts; EGF, epidermal growth factor; rh, recombinant human; β-gal, β-galactosidase; RT-PCR, reverse transcription-PCR.
and maintained in 10% FCS containing Iscove's modified Dulbecco's medium. The rh-epiregulin and its neutralizing polyclonal antibody (derived from rabbits) were previously described elsewhere (8, 11, 12). rh-EGF was purchased from Life Technologies, Inc. A short-term effect on cell proliferation by epiregulin and its neutralizing antibody was determined by using [3H]thymidine incorporation assay. Normal fibroblasts and hTERT-BJ cells were grown in 12-well culture plates with 70–75% confluence with 0.2% of FCS containing medium for 24 h and then subject to various treatments for another 24 h. Four μCi of methyl-[3H]thymidine was then added into each well overnight. Cells were transferred to a 96-well plate, and the radioactivity was measured in a 1450 Microbeta Trilux (Wallach, Sweden). To examine the effect of epiregulin on anchorage-independent growth of BJ cell transformed by hTERT in combination with SV40 and H-ras, a soft agar colony formation assay was used. The cells were cultured on soft agar in the absence or presence of epiregulin-neutralizing antibody at 10 μg/ml, and the number of colonies was counted at day 12 after seeding. To investigate the long-term effect of epiregulin neutralization of hTERT-BJ cells, cells were maintained in medium with 1% of FCS and 10 μg/ml rabbit polyclonal antibody against epiregulin, and cell number and viability were recorded.

Microarray Experiments. Actively proliferating (<30 population doublings), normal fibroblasts (nontransduced), and hTERT-BJ cells under the same culture condition were subject to total cellular RNA extraction and mRNA enrichment. cDNA microarray production was performed within the Van Andel Institute Laboratory of Microarray Technology (13). Briefly, 20,736 cDNA clones were PCR amplified directly from bacterial stock purchased from Research Genetics (Huntsville, AL). After ethanol precipitation, cDNA clones were dissolved in appropriate amounts of water. The sample was incubated with 100 μl of 0.5 M sodium acetate, pH 5.0, 3 μl of 1 M sodium hydroxide and 3 μl of 3 M sodium acetate, pH 5.0. After incubation at room temperature for 20 min, the mixture was centrifuged at 15,000 rpm for 5 min, followed by 2 × 100 μl of 1 M sodium acetate, pH 5.0, 1 ml of 100% ethanol, and 1 ml of 70% ethanol. After centrifugation at 15,000 rpm for 5 min, the sample was dried at 37°C for 15 min. The sample was dissolved in 10 μl of TE (100 mM Tris-HCl, 1 mM EDTA, pH 8.0). 1 μl of 10% dithiothreitol (DTT) was added to the sample before use for hybridization.

Microarray Data Analysis. Image files were analyzed using GenePix Pro 3 image analysis software (Axon Instruments). Spots showing no signal or obvious defects were discarded. The local background was subtracted from the net fluorescence from the Cy3-specific channel was calculated for each remaining spots, and the ratio of fluorescence from the Cy5-specific channel to the red spot, representing mRNA expression in hTERT-BJ cells relative to that in normal fibroblasts. The microarray experiment was performed three times and with reciprocal labeling to control for differential labeling efficiencies associated with the specific dyes used. The microarrays were normalized with a factor calculated from dividing one by the median ratio of all spots on each array.

RT-PCR for hTERT and Epieregulin mRNA. cDNA synthesis and PCR for hTERT mRNA were described elsewhere (14). The PCR primer set specific for epiregulin was as follows: 5'-gtggtgtgaacacagatg-3' (forward) and 5'-cagtcatttggtctctgctaa-3' (reverse). PCR reaction was performed within a linear phase by using 30 cycles (95°C at 15 s, 55°C at 60 s, and 72°C at 90 s), and the PCR products were separated on 2% agarose gels. Four μCi of [3H]thymidine was then added into each well overnight. Cells were transferred to a 96-well plate, and the radioactivity was measured in a 1450 Microbeta Trilux (Wallach, Sweden). To examine the effect of epiregulin on anchorage-independent growth of BJ cell transformed by hTERT in combination with SV40 and H-ras, a soft agar colony formation assay was used. The cells were cultured on soft agar in the presence of epiregulin-neutralizing antibody at 10 μg/ml, and the number of colonies was counted at day 12 after seeding. To investigate the long-term effect of epiregulin neutralization of hTERT-BJ cells, cells were maintained in medium with 1% of FCS and 10 μg/ml rabbit polyclonal antibody against epiregulin, and cell number and viability were recorded.

Intimate Relationship between Epieregulin Activation and hTERT-mediated Immortalization and Transformation. One of the most striking expression differences was the high level of epiregulin mRNA in hTERT-BJ cells compared with its absence in normal fibroblast, as demonstrated by both microarray and RT-PCR assays (Fig. 1). To investigate whether this is a general phenomenon for hTERT-immortalized fibroblasts, we measured the epiregulin expression in hTERT-immortalized dermal fibroblasts from another individual. As shown in Fig. 2A, epiregulin mRNA could not be detected in the primary fibroblasts, however, introduction of hTERT into these cells significantly induced expression of epiregulin. Moreover, hTERT/SV40/H-ras-transformed human BJ fibroblasts generated in another laboratory (9) similarly exhibited transcriptional activation of the epiregulin gene. We additionally extended the analysis to include another strain of human fibroblasts, the embryonic lung fibroblasts LF1, which expresses hTERT ectopically (10). Unlike normal BJ cells, LF1 cells contained detectable levels of epiregulin mRNA, perhaps because of their embryonic origin (Fig. 2A). Nevertheless, the introduction of hTERT into LF1 cells led to an up-regulation of epiregulin expression within 20 population doublings (Fig. 2A). Taken together, the induction of epiregulin is closely related to hTERT-mediated immortalization and transformation of human fibroblasts.

We then determined the epiregulin protein expression by immunoblot...
treated with the neutralizing antibody grew slower than did the control cells, which were treated with rabbit IgG, and a difference in cell numbers became more evident with a longer culture period. During cultures of both control and treated cells, viable cells always exceeded 90% as determined by a trypan-blue exclusion, indicating that the antibody specific to epiregulin is nontoxic to the cells. hTERT mRNA expression in those cells was not affected by an exposure to the neutralizing antibody (Fig. 3C).

Induction of Premature Senescence of hTERT-BJ Cells by Epiregulin Neutralization. After incubating hTERT-BJ cells in 1% FCS (control cells) or plus 10 μg/ml epiregulin-neutralizing antibody (treated cells) for 1 month, we terminated the antibody treatment and gradually increased FCS to 6%. The control cells started to proliferate more actively in response to higher concentrations of FCS. However, no significant changes in proliferation rate were observed in the cells treated with epiregulin-neutralizing antibody, which indicates that the observed growth arrest may be irreversible (data not shown). We thus investigated whether epiregulin neutralization in hTERT-BJ cells was capable of triggering a senescence program. The cells were then analyzed for expression of β-gal, a characteristic marker for senescent cells (17). Compared with hTERT-BJ cells grown under a standard condition (10% FCS), the cells in 1% FCS exhibited a slight increase in β-gal expression (<10 versus 20% β-gal-positive cells). In striking contrast, >85% of the cells exposed to the epiregulin-neutralizing antibody were positive for β-gal staining (Fig. 4). The data suggest that the functional inhibition of epiregulin induces senescence of hTERT-BJ cells, which likely contributes to the observed growth arrest of these cells.

Inhibition of hTERT-transformed BJ Cell Growth on Soft Agar by Epiregulin Neutralization. We additionally investigated whether epiregulin affects anchorage-independent proliferation. BJ cells transformed by hTERT in combination with SV40 and H-ras were used because hTERT-BJ cells do not grow on soft agar (4, 5). The mean colony number after 12 days was 2325/10^5 control cells and 982/10^5 cells treated with the epiregulin-neutralizing antibody, respectively. This result suggests a role for epiregulin in anchorage-independent proliferation of hTERT-transformed fibroblasts.

Discussion

In this study, we determined the gene expression pattern of normal young fibroblasts and hTERT-BJ cells by using a 20,736 cDNA clone-containing cDNA microarray. Our results show that the transcriptional profile is considerably altered in hTERT-BJ cells, which might reflect important biological differences between immortalized
hTERT-BJ cells and normal mortal fibroblasts. One of the most highly up-regulated genes in hTERT-BJ cells was epiregulin, a growth factor of the EGF family (8). It seems that the activation of epiregulin is closely associated with the hTERT-induced cell immortalization because we could detect high expression levels of epiregulin in hTERT-immortalized or -transformed fibroblasts from other laboratories as well. Exogenous epiregulin accelerated cell proliferation of both normal and hTERT-immortalized fibroblasts, and the effect was similar to that of EGF. When endogenous epiregulin was neutralized with a specific antibody, hTERT-BJ cells exhibited reduced DNA synthesis and proliferation rate, replicative senescence, and inhibition of anchorage-independent growth. These data suggest that epiregulin is an important growth factor for the maintenance of infinite proliferation of hTERT-immortalized fibroblasts.

Little is known about the regulatory mechanisms behind epiregulin expression. Although there is a tight association between the epiregulin activation and hTERT expression, a transient transfection of hTERT cDNA failed to induce epiregulin expression in normal fibroblasts, suggestive of an indirect effect (data not shown). Nevertheless, epiregulin has been shown to be stringently repressed in most normal human cells while extensively activated in human tumors with infinite proliferation capability (8). In bladder cancer, high levels of epiregulin expression are associated with poor outcomes (18). Moreover, it has been observed that HCT116 colon cancer cells fail to form colonies on soft agar culture or to grow in nude mice after inactivation of epiregulin, whereas re-expression of epiregulin restores in vitro and in vivo tumorigenic capabilities of the cells (19). Taken together, aberrant epiregulin activation is likely to contribute to tumor formation and progression. The expression level of epiregulin in hTERT-BJ cells used in this study was comparable with that of HCT116 cells (data not shown). It is therefore possible that the induced epiregulin in hTERT-BJ cells may eventually drive these cells through a malignant transformation.

One may argue that hTERT-BJ cells, unlike many tumor cells, retain normal cell-cycle checkpoints, which should keep the cell growth in control even in the presence of abnormal mitotic stimulation. However, checkpoint pathways are functional in HCT116 cells (20), but that does not protect HCT116 cells from epiregulin-mediated tumorigenesis. Recent observations show that both induced and spontaneous malignancies significantly increased in a number of mouse models with transgenic telomerase expression, suggesting that telomerase activation alone may be sufficient to induce transformation (21, 22).

Reconstitution of telomerase activity endows somatic cells the potential of infinite proliferation, but they do not necessarily acquire an immortal phenotype because a cellular senescence program can be triggered through other pathways such as oxidative stress, oncogene activation, radiation, drugs, various hostile growth conditions, and so on (1, 10, 23). Therefore, additional changes secondary to telomerase activation may be equally important to achieve cellular immortality, e.g., epiregulin induction in hTERT-BJ cells. Indeed, alterations of growth signal pathways seem to be a common feature of human cells immortalized by hTERT. The oncogene c-Myc was found to be activated in hTERT-expressing epithelial cells (7). Keratinocytes with ectopically expressed hTERT and telomerase show a reduced expression of the p16 gene through promoter methylation (23). Inhibition of p16 expression prevents cells from growth arrest and premature senescence independently of telomere shortening (23). Furthermore, Xiang et al. (24) recently showed that the E2F pathway is activated, and cell proliferation is consequently accelerated after reconstitution of telomerase activity in lens epithelial cells. Taken together, induction of positive growth factors or repression of negative growth signals may confer hTERT-expressing cells growth advantages that prevent premature senescence induced by nontelomere mechanisms. Thus, telomerase and growth factors may work together to achieve sustained cell proliferation. All these observations additionally suggest that telomerase activation may have broad biological consequences in addition to its essential function for telomere elongation and thereby play a more important role in immortalization and tumorigenesis than previously thought.

In addition to epiregulin, considerable alterations of the expression of other positive and negative growth factors were observed, too. Several genes involved in DNA repair and epidermal differentiation were down-regulated in the hTERT-BJ cells. We also identified 51 differentially expressed ESTs. The consequences of all these differences call for further investigations.

In summary, we have identified significant differences in the gene expression profile of normal and hTERT-immortalized fibroblasts, and it is likely that these changes have important biological consequences. Some changes such as the activation of epiregulin contribute together with hTERT to the immortal phenotype of the hTERT-BJ cells but also to an increased risk of malignant transformation. With this in mind, conclusions about normal cell biology extrapolated from studies of hTERT-immortalized cells have to be approached with caution, and it seems too early to use hTERT for the expansion of normal human cells for therapeutic purposes.

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References


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