Tumor Cells Fail to trans-Induce Telomerase in Human Umbilical Vein Endothelial Cell Cultures

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Abstract

The shortening of the telomeres that occurs in most somatic cells and untransformed cell cultures is considered a hallmark of cellular senescence. Re-activation of telomerase, which is usually present in immortal cells, avoids telomere shortening and considerably extends the culture life span. Normal human endothelial cells are characterized by an accelerated rate of telomere shortening and reach replicative senescence after a limited number of cell divisions. It has recently been reported that human telomerase reverse transcriptase expression may be strongly up-regulated in human endothelial cells cocultivated with tumor cells. Due to the important implications of this finding on tumor progression, we have extensively analyzed for the presence of telomerase in primary human endothelial cell cultures either cocultivated with tumor cells or grown with tumor-conditioned medium. We found modest, but readily detectable, amounts of telomerase in all human endothelial cell cultures analyzed that disappeared as the cultures approached senescence. Quantitative reverse transcription-PCR also showed a direct correlation between human telomerase reverse transcriptase expression and the proliferative index of the cultures. Nevertheless, we did not find any evidence of induction of telomerase activity by tumor cells in any of the tested conditions. All data indicate that telomerase in human endothelial cells follows an activation program that is strictly associated to the culture growth rate.

Introduction

Endothelial cell cultures derived from human umbilical vein (human endothelial cells) represent a suitable model to investigate the mechanisms involved in endothelial cell responses to various stimuli. Normal human endothelial cells, like other somatic cells in culture, undergo a limited number of cell divisions and enter a state called replicative senescence. It is believed that a relevant factor in regulating the life span of the cells is the shortening of the telomeres, the specialized structures that seal the chromosome ends (1). Telomere shortening is contrasted by telomerase that is virtually absent in most somatic cells and tissues (2), but it is re-activated by up-regulation of the catalytic component human telomerase reverse transcriptase (hTERT) in the vast majority of tumor cells (3) and confers on them unlimited proliferative potential. Human endothelial cell cultures have a relatively high rate of telomere shortening, double that of fibroblasts, and they reach replicative senescence earlier in agreement with the telomere hypothesis. Nevertheless, telomerase has also been found in cycling cells, although it seems to be present only at early stages of subculturing (4, 5), and the replicative senescence of these cells can be bypassed by ectopic expression of hTERT with considerable extension of the culture life span (6).

Tumor cell growth induces the sprouting of vessels from division of differentiated endothelial cells with contribution of endothelial progenitors (7). When tissues grow beyond the limit of oxygen diffusion, hypoxia triggers vessels to grow by indirectly up-regulating many angiogenic genes, the most remarkable of which is the vascular endothelial growth factor (8). Recently, it has been suggested that among the factors that tumor cells release to induce the angiogenic program, there are some that re-activate the telomerase. This claim was sustained by cocultivation experiments in which it was shown that glioblastoma tumor cells induce de novo telomerase activity in human endothelial cells by diffusible factors (9).

The potentially important theoretical and practical implication of this finding induced us to extensively analyze telomerase activity in human endothelial cells cultivated up to the end of their proliferative potential and after exposure to tumor released factors. In general, we were able to detect the telomerase activity during the first two thirds of the culture life span, and we could show that hTERT expression peaks at logarithmic phases during the cell growth. On the other hand, all of our attempts to reactivate the telomerase or to up-regulate hTERT expression by tumor cells were unsuccessful, and it was so even when human endothelial cells were grown in direct contact with tumor cells or in hypoxic condition.

Materials and Methods

Cell Cultures. Pooled human endothelial cells were obtained from BioWittaker and grown in endothelial cell growth medium EGM-2 supplemented with endothelial cell Bullet kit (BioWittaker, Walkersville, MD). Three independent cultures were analyzed up to the end of their replicative potential. Appropriate numbers of cells were collected by trypsinization every 2 to 3 days at each subculturing and used for telomeric repeat amplification protocol (TRAP) and reverse transcription-PCR analysis. To obtain resting cells, the cultures were maintained for 3 to 4 days after confluence either in growth medium or in Dulbecco’s modified Eagle’s medium plus 2% serum. Cocultures of human endothelial cells and T98G human glioblastoma cells or primary skin human fibroblasts were obtained using trans-well polycarbonate membrane polystyrene plates (pore size, 0.4 μm; Corning Costar Inc., New York, NY). Cells were plated at a density of 10^4 cells/cm^2 in separate compartments and maintained in EGM-2 complete medium. Cultures were analyzed at subconfluence and after being cocultivated for three more passages. In some experiments, tumor cell-like U937 and SK-MEL-28 were used instead of T98G. For the experiments with conditioned medium, T98G and human fibroblast cells were cultured for 4 days in complete endothelial cell growth medium, EGM-2. Conditioned media were collected and either centrifuged at 1500 rpm for 5 minutes or filtered through 0.4-μm pore size filter and used to maintain human endothelial cells in culture for 3 days. Cultures of T98G and human endothelial cells grown in RPMI and EGM-2, respectively, for the same time intervals were used as controls. ALT telomerase-negative tumor cell lines U2OS or SAOS-2 were admixed with cultures of human endothelial cells that had been seeded the day before. In other experiments, the two types of cell were seeded simultaneously and grown together for 3 days before sample collection. Hypoxic conditions were obtained as described.
An aliquot of 3 primers, and 2 U of in human endothelial cells was also compared with that found in HeLa cells. The amount of hTERT found in cultures of human endothelial cells was also compared with that found in HeLa cells. Relevant band to the intensity of the GPDH band. The amount of hTERT found in human endothelial cells was also compared with that found in HeLa cells.

Quantitative Reverse Transcription-Polymerase Chain Reaction. Total RNA was extracted from 2 × 10⁶ human endothelial cells with the TRIZOL LS reagent (Invitrogen, Carlsbad, CA). Total RNA (10 µg), DNase pretreated, was reverse-transcribed by random hexamers, and one fifth of the resulting cDNA was used. GPDH and hTERT fragments were coamplified using the following primer sets: GPDH/F (CACAGTCCATGCCATCAC) and GPDH/R (CACACCCCTTGTGGCTGTA), and hTERT 1784 (CAGAAGATGCTGAGGCA) and R/hTERT 1928 (GGATGAAGCGGATCTGGA; ref. 12). The amplification was performed in a mixture of 50 µL containing 1.5 mMol/L MgCl₂, 200 µmol/L dNTPs, 20 pmol of TERT primers, 5 pmol of GPDH primers, and 2 U of Taq polymerase. The PCR reaction was conducted for 30 cycles with the following settings: 94° for 20 seconds, 60° for 20 seconds, and 72° for 20 seconds. The amplified products were separated on 6% nondenaturing polyacrylamide gels and stained by ethidium bromide. The relative amount of hTERT transcript was calculated by normalizing the intensity of the relevant band to the intensity of the GPDH band. The amount of hTERT found in human endothelial cells was also compared with that found in HeLa cells.

Results

Three independent human endothelial cell cultures (A, B, and C) were followed up to their replicative senescence. A quantitative reverse transcription-PCR was set up to measure hTERT expression more accurately. Specific regions of hTERT and GPDH genes were coamplified. The optimal amplification conditions to detect variable amounts of hTERT were determined using different amounts of HeLa RNA mixed to a fixed amount of fibroblast RNA (data not shown).

As shown in Fig. 1, telomerase activity can be detected in growing cells independently of the growth rate of the cells, but not in quiescent confluent cultures. On the other hand, the level of hTERT expression was maximum in cultures 70 to 90% confluent and totally absent only in cultures maintained for 4 days in 2% fetal calf serum minimal essential medium after confluence. Although the level of hTERT expression was minimal, it was still detectable in cultures maintained in growth medium for 3 to 4 days after confluence. The highest level of expression in human endothelial cells was about one third of that routinely observed in HeLa cells (Fig. 1B).

We then tested subconfluent cultures until they exhausted their proliferative potential. We obtained 44 cell doublings for culture A and 35 and 40 cell doublings for cultures B and C, respectively. As shown in Fig. 2, telomerase activity was detected up to 29, 23, and 26 cell doublings for cultures A, B, and C, respectively. In all TRAP-positive samples, we detected a constant level of hTERT expression. Although at much lower level, hTERT was still detectable for an additional two or three cell doublings after the last TRAP-positive sample (Fig. 3).

Cocultivation experiments were set up with cultures in which telomerase was either easily detectable or absent and with cultures in which only hTERT transcript was detectable. The cells were exposed to factors released by tumor cells either by cocultivating human endothelial cells and tumor cells in separate compartments of trans-well plates or by growing human endothelial cells in tumor conditioned medium. Control experiments in which tumor cells were substituted by normal human fibroblasts were also performed. To determine whether cell–cell contact could potentially induce or up-regulate telomerase, human endothelial cell and telomerase-negative tumor cell lines that have developed alternative mechanisms of telomere maintenance were grown in the same flask.

Typical results are shown in Fig. 4. In no case and with none of the conditions tested did we see changes in telomerase activity of human endothelial cells. On the other hand, the quantitative analysis showed a significant reduction of hTERT transcript in human endothelial cells...
in which only hTERT was detectable. In general, we found that the amount of hTERT in cells exposed to tumor-released factors was about one half of that present in unexposed control cultures. Similar results were obtained with human endothelial cells exposed to fibroblast and to different tumor cultures, such as U937 and melanoma cell lines. This result was obtained also in the experiments in which human endothelial cells and tumor cells were grown with intimate contact conditions (Fig. 4C).

While this manuscript was under revision, it was shown that the hTERT promoter has hypoxia-inducible factor 1 response elements (13). It is conceivable that in the hypoxic environment of a tumor, hTERT could be maintained or up-regulated in human endothelial cells. Thus, we set up experiments for culturing human endothelial cells that have low or no hTERT or telomerase in hypoxic conditions either with growth medium (Lane 2) or with tumor conditioned medium (Lane C), indicating that up-regulation of telomerase is more likely a general mechanism that is coupled to fast-cycling endothelial cells (ref. 17; this paper).

In their paper, Falchetti et al. (9) described experiments in which tumor-released factors induced hTERT expression and telomerase activity in young human endothelial cell cultures while control unexposed cultures were and remained negative. The reactivation of telomerase has been described to spread throughout the culture starting from isolated cells, and only after 5 days, most of them expressed telomerase. This observation is in conflict with the result expected if telomerase is activated by diffusible factors. It is likely that all of the cells in the culture would be simultaneously and equally exposed, and they should up-regulate telomerase in a relatively limited time interval.

However, none of the experiments that we carried out, either with telomerase-positive or telomerase-negative human endothelial cells, resulted in telomerase up-regulation. Telomerase-negative human endothelial cells remained such even if the cocultivation experiments

Discussion

High levels of telomerase activity appear to be critical in maintaining stable telomere length. Nevertheless, telomerase activity has been detected in normal cells that show telomere shortening as well (14). In these cells, telomerase activity is considered insufficient to sustain telomere length. Human endothelial cell cultures seem to have an unusual behavior. They present easily detectable telomerase activity until they reach the last one third of their replicative life span. Successively, the level of expression of hTERT dramatically drops to less than one tenth of its typical peak expression (it is noteworthy that the culture growth rate strongly slows down at this stage), but the telomeres keep shortening constantly and the rate of shortening is double that of fibroblasts in which telomerase levels are much lower (15). Moreover, hTERT expression is higher in fast cycling human endothelial cells, although it is only one third of that found in HeLa and very low or virtually absent in quiescent cells.

Because the sprouting of new vessels is driven by the activation of endothelial cell division, it is understandable that the hTERT expression can become consistent during early steps of angiogenesis. The presence of hTERT in endothelial cells of vessels that nourish tumors like glioblastoma multiforme was the first observation to suggest that tumor cells may specifically trans-induce telomerase (16). On the other hand, telomerase re-activation in endothelial cells was also shown in granulation tissue in wound healing of the human skin,
were prolonged up to three subcultures. In contrast, the quantitative analysis of hTERT showed that the cells exposed to tumor factors showed considerably lower levels of transcript than control cultures. It is reasonable to suppose that cells growing in conditioned medium experienced a reduction of the proliferation rate and consequently of the telomerase expression.

It is very unlikely that the conflicting results obtained by us are due to nonidentical experimental conditions. In addition, to perform the experiments tightly to the description, other conditions were investigated. Furthermore, experiments with human endothelial cells at a different moment of their replicative history were repeatedly exploited. The results obtained with cultures in which only hTERT was detectable are particularly meaningful. Under these conditions our quantitative analysis clearly shows a decrease of the amount of hTERT after cocultivation. More importantly, we also show that even if tumor cells are grown in the same flask as human endothelial cells, so that cell to cell contacts are present, we do not have telomerase reactivation, and the amount of hTERT clearly decreases. Finally, telomerase was reactivated or up-regulated neither by hypoxia nor by culturing human endothelial cells with tumor conditioned medium in hypoxic conditions.

In conclusion, our data do not support the hypothesis that tumor cells in culture are able to trans-induce telomerase in human endothelial cells. In these cells, telomerase activity seems to be strictly coupled to the cell proliferation. Thus, all conditions that have a detrimental effect on the culture growth rate parallel to down-regulation of telomerase activity. On the other hand, the main role of telomerase in the endothelial cells does not seem to be devoted to avoid telomere shortening.

References
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