Telomerase-negative Immortalized Human Cells Contain a Novel Type of Promyelocytic Leukemia (PML) Body

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Abstract

Telomerase-negative immortalized human cells maintain their telomers by a mechanism known as alternative lengthening of telomeres (ALT). We report here that ALT cells contain a novel promyelocytic leukemia (PML) body (ALT-associated PML body, APB). APBs are large donut-shaped nuclear structures containing PML protein, telomeric DNA, and the telomere binding proteins human telomere repeat binding factors 1 and 2. Immunostaining showed that APBs also contain replication factor A, RAD51, and RAD52, proteins involved in DNA synthesis and recombination. During immortalization, APBs appeared at exactly the same time as activation of ALT. APBs were found in ALT tumors and cell lines but not in mortal cell strains or in telomerase-positive cell lines or tumors.

Introduction

Structures seen in the normal interphase nucleus include nucleoli, coiled bodies, and PML bodies (1). The latter are donut-shaped nuclear domains containing PML protein (2). In acute PML, the PML protein forms a fusion protein with retinoic acid receptor α (RARα) due to a chromosome 15:17 translocation (3). Presence of the PML-RARα fusion protein disrupts PML bodies in acute PML cells, and treatment with retinoic acid restores PML bodies via an unknown mechanism. A number of additional proteins have been found to reside in PML bodies, including CBP, SUMO-1, and pRb (4–6). The function of PML bodies is not presently known but may include a role in tumor suppression. Recently, PML protein has been shown to play a direct role in the apoptotic pathway (7, 8) and was found to participate in the control of MHC class I antigen presentation (9).

Some immortalized human cell lines have no detectable telomerase activity and maintain their telomers by an ALT mechanism (10). Some telomerase-negative tumors also use ALT (11). Here we describe evidence that ALT cell lines and tumors contain a novel form of PML body in which PML protein colocalizes with telomeric DNA and the telomere binding proteins hTRF1 and hTRF2. These structures were not detected in mortal cells or in telomerase-positive cell lines and tumors and are, therefore, referred to as APBs. In a cell line immortalized in vitro, APBs became detectable at the same PD level at which the TRF pattern characteristic of ALT cells was first seen. In view of the possibility that the ALT mechanism may involve recombination (12), it is interesting that these novel nuclear bodies also contain replication factor A, RAD51 and RAD52, proteins involved in recombination and other aspects of DNA metabolism (13).

Materials and Methods

Cells. The cell strains and cell lines used in this study include the WM1175 malignant melanoma and HUT292DM lung carcinoma cell lines and those listed in Table 1. Culture conditions have been described previously (10, 11, 14).

FISH. Cells were fixed with methanol-acetic acid (20°C for 6 min) and acetone (~20°C for 30 s). The primary antibodies used included rabbit polyclonal hTRF1, hTRF2 (from T. de Lange), RAD51 (Ab-1; Oncogene Research Products, Cambridge, MA), PML antibody 2912A (4), mouse monoclonal PML (PG-M3; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), replication factor A (Ab-1; Oncogene Research Products), and mouse polyclonal RAD52 (from Dr. Z. Shen) and were detected with either FITC- or Texas Red-conjugated secondary antibodies (Sigma, St. Louis, MO or Vector Laboratories Inc., Burlingame, CA).

GFP Plasmid. A plasmid encoding a GFP-hTRF1 fusion protein (from T. de Lange) was transfected into GM847 and HeLa cells with LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD). G418-resistant clones were picked, and GFP fluorescence was detected in living cells by fluorescence microscopy.

Tumors in Nude Mice. The IIICF/c fibroblast cell line (ALT) was transfected with pSVneo-EJras plasmid (containing the activated c-Ha-ras oncogene from the EJ bladder carcinoma cell line) DNA, selected with G418, and injected s.c. into nude mice to obtain ALT tumors. Telomerase-positive nude mouse tumors were obtained by injecting nude mice with WM1175 (malignant melanoma) and HUT292DM (lung cancer) cells.

Paraffin Sectioning and Antigen Retrieval. Human tumors and nude mouse tumors formed by human cell lines were fixed with 2% paraformaldehyde and embedded in paraffin. Tumors were then sectioned and dewaxed. For antigen retrieval, the sections were heated at 100°C in 0.01 M Tris buffer (pH 10.0) for 10 min in a microwave oven (16). IHC was performed with either the rabbit polyclonal anti-hTRF1 antibody or anti-PKCδ (Santa Cruz Biotechnology, Inc.) as a negative control.

TRF Analysis. Genomic DNA was digested with the restriction enzymes HinfI and RsaI and electrophoresed in a 1% agarose pulsed field gel as described previously (10). A χ32P-labeled (TTAGGG)3 probe was used to detect the telomere signal.

TRAP Assay. The PCR-based TRAP assay (17) was used to detect telomerase enzyme activity.
 unexpected, FISH analysis of ALT cell lines (TRF length found by Southern analysis to be characteristic of ALT) observation correlates well with the highly heterogeneous telomere were identified (data not shown), as documented previously (18). This a probe for TTAGGG telomere repeat DNA, telomeres of greatly

Results

When we examined metaphase spreads of ALT cells by FISH with a probe for TTAGGG telomere repeat DNA, telomeres of greatly varying lengths ranging from short or undetectable to abnormally long were identified (data not shown), as documented previously (18). This observation correlates well with the highly heterogeneous telomere TRF length found by Southern analysis to be characteristic of ALT cells (10). Unexpectedly, FISH analysis of ALT cell lines (e.g., GM847; Fig. 1a) also revealed brightly staining nuclear bodies detectable by TTAGGG repeat DNA probes in a subpopulation (~5%) of the interphase cells. The remaining interphase cells did not have large nuclear bodies but instead had a sprinkled staining pattern of their telomeres. FISH was also performed with a telomere-specific peptide nucleic acid probe, and the results were identical to those seen with the plasmid DNA probe (not shown).

The presence of telomeric repeat DNA in these nuclear aggregations prompted us to examine whether they also contained the telomere binding protein hTRF1 (19). IHC staining using an anti-hTRF1 rabbit polyclonal antibody showed that hTRF1 protein colocalized with telomeric DNA in the large nuclear structures (Fig. 1b). IHC staining with this antibody produced a sprinkled pattern indistinguishable from that seen with telomere FISH in the remaining interphase nuclei (not shown).

To eliminate the possibility that the nuclear aggregations were an artifact of the fixation and staining conditions, we stably transfected GM847 (ALT) and HeLa (telomerase-positive) cells with a plasmid encoding an hTRF1-GFP fusion protein. The cells were selected with G418, and then unfixed cells were examined by fluorescence microscopy. The results (Fig. 1c) were the same as seen with anti-hTRF1 IHC in fixed cells; there was a subpopulation of GM847 (but not HeLa) interphase cells containing large nuclear aggregates of the GFP fusion protein. When the transfected GM847 cells were subsequently processed for detection of telomeric DNA by FISH, it was found that the green fluorescence colocalized with aggregates of telomeric DNA (not shown).

To determine whether the nuclear aggregates are seen in ALT tumors as well as in ALT cell lines in vitro, we first generated ALT tumors by transfecting the nontumorigenic ALT cell line, IIICF/c (20), with an activated c-Ha-ras oncogene, and injected the cells into athymic nude mice. The resulting tumors had no detectable telomerase activity in the TRAP assay, and Southern analysis showed that they retained the TRF length pattern diagnostic of ALT (not shown). IHC staining of tumor frozen sections by anti-hTRF1 IHC showed that nuclear aggregates were readily detected in a subpopulation of the tumor cells (not shown). When the tumors were embedded in paraffin, sectioned, and subjected to antigen retrieval, anti-hTRF1 IHC revealed the nuclear aggregates of hTRF1 in the ALT tumors (Fig. 1d) but not in tumors formed by two telomerase-positive cell lines, WM1175 and HUT292DM (not shown). A human breast carcinoma specimen that had previously been shown to be telomerase-negative and to have the TRF length pattern diagnostic of ALT [tumor 334 (11)] was paraffin-embedded, sectioned, and stained for hTRF1 after antigen retrieval. The nuclear aggregates were clearly visible in a subpopulation of the breast cancer cells (Fig. 1d).

To further examine the correlation between ALT and the nuclear aggregates, we examined a number of immortalized cell lines and mortal cell strains (Table 1). In telomerase-positive cell lines such as HeLa (Fig. 1e), nuclear aggregates containing hTRF1 were not detectable, and only the sprinkled pattern was visible. Telomerase-negative mortal cells, either normal or transformed but nonimmortalized, had the same staining pattern as telomerase-positive cells (Table 1). Each of 12 ALT cell lines was found to have a subpopulation containing the nuclear aggregates. One of the ALT lines, WI38-VA13/2RA, contained nuclear aggregates that were intermediate in size between the nuclear sprinkles found in the telomerase-positive cell lines and the nuclear aggregates found in the other 11 ALT lines (Table 1).

In view of the possibility that the ALT mechanism involves a recombination step (12), we also stained the cells with antibodies against proteins involved in recombination and showed that the hTRF1 nuclear aggregates colocalized with RAD52 (Fig. 1, f and g) and replication factor A (Fig. 1, i and j). Some of the nuclear aggregates detected by immunostaining for each of these three proteins were denser at the periphery than at the center (Fig. 1, g and j). In retrospect, this donut-shaped appearance could also be seen in the aggregates stained with the anti-hTRF1 antibody (Fig. 1, a and k).

The nuclear aggregates were clearly separate from the nuclei (e.g., Fig. 1, g and h), and because PML bodies are often donut shaped, we used anti-PML antibodies to determine whether the aggregates contain PML protein. hTRF1 was shown to colocalize with PML protein in the nuclear aggregates (Fig. 1, k and l). Although the hTRF1 aggregates all colocalized with PML, hTRF1 could not be detected in some PML bodies (not shown), indicating that the nuclear bodies containing telomeric DNA and telomere-specific binding protein are a subset of PML bodies. Similarly, RAD52 (Fig. 1, m and n), replication factor A (Fig. 1, o and p), the telomere binding protein hTRF2 (Fig. 1, q and r), and RAD51 (Fig. 1, s and t) also colocalized with PML in these nuclear bodies. The nuclear aggregates present in the ALT cell lines are thus a novel form of PML body and are, therefore, referred to below as APBs.

ABPs were found in immortalized IIICF/c cells, but not in their preimmortalized counterparts (Table 1). To determine when APBs first appear, we examined a newly generated ALT cell line. For unknown reasons, cells from individuals with Li-Fraumeni syndrome have mostly given rise to ALT cell lines; therefore, to maximize the probability of obtaining an ALT line, we used IIICF Li-Fraumeni syndrome fibroblasts. IIICF fibroblasts became senescent at PD40 (20), but after 6 weeks, some cells in a flask designated IIICF/a2 underwent growth arrest accompanied by morphological changes suggestive of senescence or crisis, but
within 30 days, the culture was overgrown by rapidly proliferating cells, consistent with immortalization having occurred (Fig. 2A).

Genomic DNA was extracted from the cells at various PD levels, and the TRF length was determined. The telomeres were short up until PD76, with slight shortening of the major TRF band being seen between PD70 and PD76 (Fig. 2B). From PD76, the cells were found to have the heterogeneous TRF length (ranging from short to extremely long), characteristic of ALT cell lines (Fig. 2B). IIICF/a2 had no detectable telomerase activity in the TRAP assay, either before or after telomere lengthening occurred (not shown). Thus, it is clear that the ALT mechanism was activated between PDs 76 and 77.

Cells at each of the PD levels shown in Fig. 2B were also immunostained with anti-hTRF1 antibodies to determine the point at which APBs appeared. From PD70 to PD76, APBs were seen rarely, and from PD77 to PD142, they were easily visualized, occurring in ~5% of the population (Fig. 2C). This is consistent with the existence of a minor cell subpopulation in which ALT was already activated prior to PD76 and which rapidly overgrew the culture when the majority of cells entered growth arrest. This indicates a very tight temporal correlation between the activation of ALT and the occurrence of APBs.

**Discussion**

These data show that a subpopulation of ALT cells have donut-shaped nuclear domains we refer to as APBs, containing not only PML protein but also telomeric DNA and the telomere-specific binding proteins hTRF1 and hTRF2. Consistent with the finding that some telomeric DNA sequence in ALT cells is present in nuclear domains distinct from telomeres, others have detected extrachromosomal tracts of telomeric DNA in ALT cell lines but not in telomerase-positive cell lines (21, 22). In addition, these nuclear bodies contain RAD51, RAD52, and replication factor A, proteins involved in DNA processing. These novel PML bodies were seen only in ALT cell lines, and their appearance coincided with activation of the ALT mechanism during *in vitro* immortalization.

APBs provide a simple marker for ALT. To test cell lines or tumors for the presence of ALT, it has been necessary until now to obtain high molecular weight genomic DNA for TRF analysis and protein lysates from samples either freshly obtained or stored at or below −80°C for the TRAP assay. This precluded the use of tumor samples which had been fixed and paraffin embedded. The ability to detect APBs in paraffin sections after antigen retrieval will make it possible
to analyze a wide variety of archival tumor material for the presence of ALT and to facilitate analysis of its prognostic significance.

With PML recently shown to be involved in apoptosis, another possibility might be that APBs appear in ALT cells that are destined to undergo apoptosis, e.g., due to failure to adequately maintain their telomeres. If this is the case, APBs still appear to be specific for ALT cells because preliminary studies have shown that some telomerase-positive cells induced to undergo apoptosis do not contain APBs (data not shown).

An obvious feature of APBs is that although they were found in all of the ALT lines examined, within each ALT cell line they were detected in only a subset (~5%) of the interphase nuclei. A possible explanation might be that APBs are only formed in a particular phase of the cell cycle or in cells that have exited the cell cycle. Preliminary data indicate that many of the APB-containing cells do not have senescence-associated β-galactosidase activity and are, therefore, unlikely to be senescent. Another possibility might be that APBs represent reservoirs of telomeric DNA and associated proteins required in cells actually undergoing telomeric maintenance. The relationship between APBs and the small circular DNA molecules containing telomere repeat sequence found recently in some immortalized cell lines (23) needs to be clarified. APBs could also be staging platforms for the maintenance process, e.g., facilitating recombination between telomeres. Alternatively, they may be by-products of the telomere maintenance process that have not yet been degraded or recycled.

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