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Irreversible Telomere Shortening by 3′-Azido-2′, 3′-Dideoxythymidine (AZT) Treatment

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Telomeres shorten by 30 to 50 bp with each cell division. Germ line, tumor and stem cells overcome progressive shortening by elongating their telomeres with telomerase. Previously we demonstrated that 3′-azido-2′,3′-dideoxythymidine (AZT), incorporates into telomeric DNA. To determine if telomeric AZT incorporation was a telomerase mediated phenomenon, we subjected tumor cells to long-term AZT exposure. Here we report the shortening of the telomeric sequences of HeLa cells cultured with 800 μM AZT for 15 passages. Southern blots of HeLa DNA cultured with AZT and digested with SAU 3AI, Alu I, and Rsa I revealed a progressive shortening of the telomeric repeats when probed with a human biotinylated telomeric probe. The shortened telomeric repeats did not elongate after culturing without AZT for an additional 25 passages. No evidence of senescence could be detected.

Normal human diploid cells in culture, divide a certain amount of times and enter a nondividing state termed senescence. Telomere loss is thought to control entry into senescence. Telomeres consist of specific repeats at chromosome ends which are synthesized by a reverse transcriptase called telomerase (1). Telomerase is active in germline cells and not expressed in most human somatic tissues. In contrast, in most tumors telomerase activity is detectable (2). Recent data indicate that activation of telomerase in human tumors bypasses cellular senescence and is thus a requirement for tumor progression (1). Telomere maintenance is a prerequisite for continued tumor growth, therefore telomere shortening is a tumor suppressing mechanism (3). In a previous report we demonstrated the preferential incorporation of AZT in telomeric sequences of CHO cells, indicating that such incorporation could be telomerase-mediated (4). In this report we subject HeLa cells to long term exposure with AZT to evaluate if telomere shortens and if so, the reversibility of the phenomenon.

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MATERIAL AND METHODS

Cell culture and AZT exposure. HeLa cells (ATCC, Rockville, MD) were cultured in 75 cm^2 flasks (Costar, Cambridge, MA) with minimum essential medium Eagle (EMEM, Bio Whittaker, Walkersville, MD) supplemented with 8% fetal bovine serum (Intergen, Purchase, NY) and antibiotics. Cells were plated at a 25% confluence and subcultured when they reached 100% confluence (2 to 5 days). To all cultures 800 μM AZT (Sigma Chemical Co., St Louis, MO) was added. After culturing 15 passages with AZT, half of the flasks were switched to AZT free media and labeled with the letters "PR", indicating the number of passage after removal.

Cell replication. To determine the number of cell divisions per passage, HeLa cells were grown in EMEM containing 1 μCi/ml [^{3}H]-thymidine (25 Ci/mmol, Amersham, Arlington Heights, IL) for 72 hours. Then, the cells were transferred to 6-well plates and cultured in EMEM medium without [^{3}H]-thymidine. Radioactivity of DNA isolated from cells at the time of the transfer was considered "Time 0" and any dilution of the radioactive label after 24 hours of culture was considered to be the result of DNA replication.

DNA extraction and restriction. Cells from each passage were collected and high molecular weight DNA was extracted using a non-organic extraction procedure (Oncor, Gaithersburg, MD). DNA was digested with restriction endonucleases Alu I, Rsa I, and Sau 3A1 (New England Biolabs, Beverly, MA) for 2 hours at 37°C.

Southern blotting. Digested HeLa DNA was resolved in a 1% agarose gel along with a digoxigenin-labeled molecular marker (Molecular Marker III, Boheringer Mannheim, IN). The DNA was transferred to a nylon support membranes overnight, by capillary action and UV-cross-linked to the membranes. Membranes were blocked for 30 min at 45°C with blocking solution (Oncor) and hybridized with a digoxigenin-labeled human telomeric probe (200 ng/ml Hybrisol III, Oncor) overnight in a sealed bag at 45°C. Post-hybridization washes were performed in a solution of 0.16 × SSC/0.1% SDS for 1 hr at 60°C. The membranes were then blocked with a 5% low fat milk solution in TBS-Tween (0.05 M Tris-HCl, 0.15 M NaCl, 0.2% Tween 20, pH 7.5) at room temperature for 30 min. An antidigoxigenin antibody alkaline phosphatase conjugated, (Boheringer Mannheim) was diluted to 1:3000 in 5% low fat milk solution in TBS-Tween and

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used to incubate the membranes for 30 min with agitation. After washes in TBS-Tween for 20 min the membranes were stained using 200 μl of the alkaline phosphatase substrate, 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt, (BCIP), together with Nitro blue tetrazolium chloride (NBT) (NBT/BCIP stock solution Boheringer Mannheim) diluted in 10 ml of staining solution (0.1 M Tris-buffer, 0.05 M $MgCl_2$, 0.1 M NaCl, pH 9.5). The membranes were immersed in staining solution at 37°C until blue color developed, rinsed with distilled water, dried with filter paper, and photographed.

Fluorescence in situ hybridization (FISH). Passage 13 HeLa cells cultured either with or without AZT were grown in Labteck chambers. Cells were fixed in formalin and denatured with a 70% formamide solution at 70°C for 2 min. The slides were incubated overnight with a biotinylated telomeric probe (14.8 ng/slide, Oncor) and washed with a post-hybridization solution containing 50% formamide. Detection of the telomeric biotinylated signal was achieved with fluorescein-labeled avidin (Oncor) and the nuclei were counterstained with propidium iodide. Slides were photographed with a Nikon Labphot microscope equipped with an epifluorescence (HMX-HBO-100w lamphouse) attachment. A double pass filter was used to capture simultaneously the propidium iodide and fluorescein signals. Kodak Ektachrome (ASA 400) daylight film was used for immunofluorescence photography.

RESULTS

[^{3}H]-thymidine-DNA quantification, by dilution of the label due to cell replication, revealed that 1.1 cell replication rounds took place every 24 hours in the control cells, whereas 1.5 and 1.8 cycles took place in the treated and recovered cells, respectively. DNA from HeLa cells cultured with AZT for 0, 4, and 15 passages probed with a digoxigenin human telomeric probe is shown in Fig. 1, lanes 1 to 3. Lanes 4 and 5 show respectively DNA from cells growing in AZT free medium for 6 or 25 passages after AZT exposure for 15 passages. Lane 6 shows a digoxigenin molecular marker (MM). A decrease in the telomeric fragment size was observed with increasing passage number and was irreversible once AZT was removed from the media even after 25 passages (Figs. 1 and 2). The shortened telomeric length was indicated by a longer area hybridizing with the telomeric probe (Fig. 1) and by a decreased in the mean terminal restriction fragment (TRF) length (Fig. 2). Fluorescence *in situ* hybridization was also performed in cells growing with or without AZT (Fig. 3). Bright spots in Fig. 3 represent hybridization of fluorescent telomeric probes to the telomeres. A weaker signal was observed in cells exposed to AZT (Figure 3A) as compared with untreated cells (Fig. 3B). Markers of senescence such as p21 (Waf1), small proline rich proteins (spr), and apoptosis, were analyzed after AZT treatment. The results were indistinguishable from the control untreated cells or the recovered cells. (Data not shown). Therefore, no signs of morphological or biochemical changes attributable to senescence were observed.

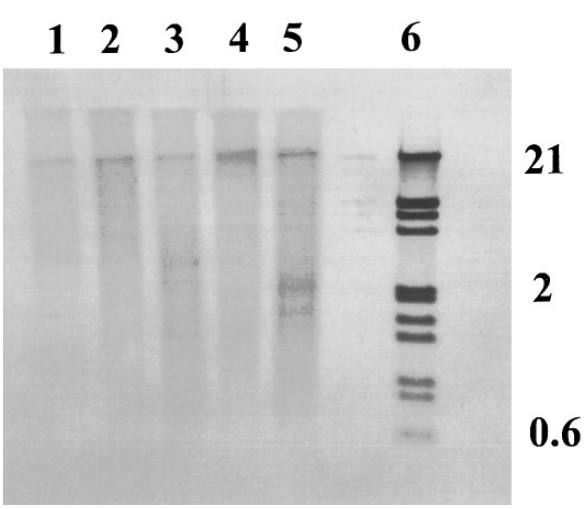

FIG. 1. Southern blot. Lane 1: untreated HeLa cells. Lanes 2 and 3: AZT treated HeLa cells at passages 4 and 15 respectively. Lanes 4 and 5: HeLa cells growing after AZT removal for 6 and 15 passages respectively. Lane 6: digoxigenin molecular marker. The signal specific for telomeric repeats, reveals a shortening in the size of the repeats with time of exposure and reveals no changes in the length of the repeats after the cells have been allowed to grow for 6 or 25 passages without AZT.

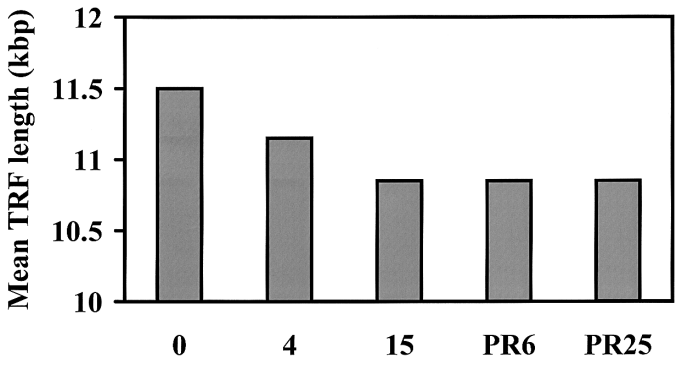

FIG. 2. Mean terminal restriction fragment (TRF) length of DNA expressed in kbp for HeLa untreated cells (0), AZT treated for several passages (4 and 15) and cells cultured after AZT removal (PR6 and PR25).

DISCUSSION

Telomere repeats are larger in embryonic life, as compared with adults, because approximately 50 bp are lost in each cellular division (5). When the shortening reaches a particular length the cell stops dividing, initiates a program of senescence, and dies (6). Embryonic cells, malignant cells, and immortalized cells avoid telomere shortening by replicating their chromosomal ends with telomerase (7). Telomerase, an unusual reverse transcriptase, adds *de novo* repeats to the ends of the chromosomes (8), and is likely to be involved in incorporation of AZT, as a chain terminator, into the newly synthesized DNA in place of thymidine. It is known that AZT has a low affinity for DNA polymerase

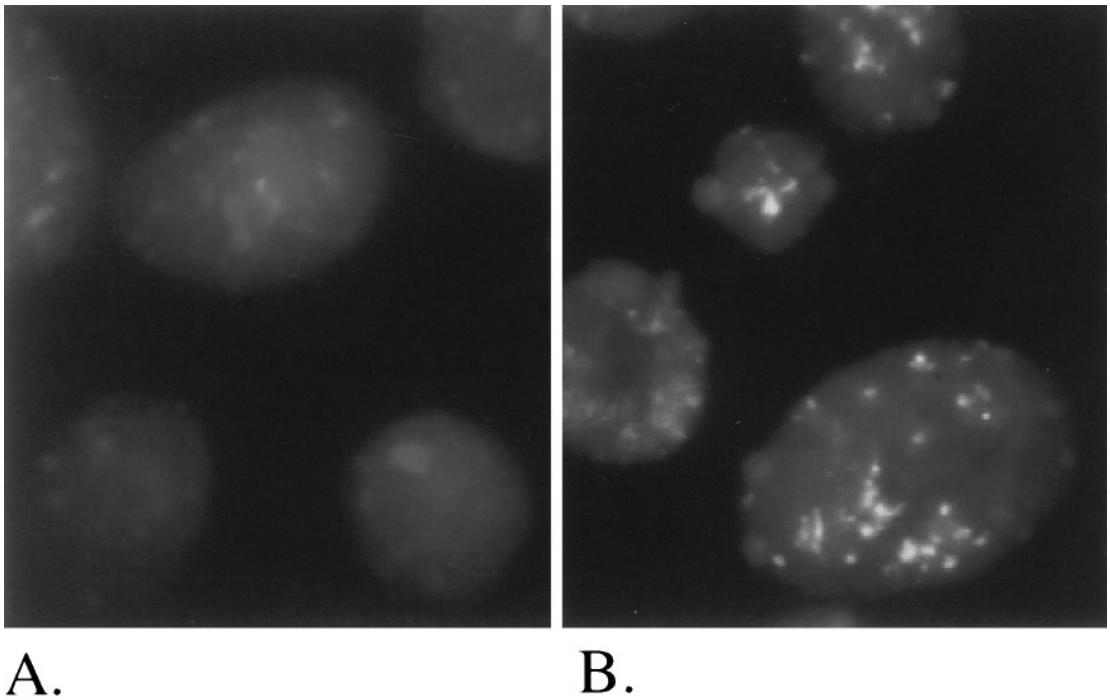

FIG. 3. Non quantitative fluorescence *in situ* hybridization of HeLa cells with a biotinylated telomeric probe: A. Interphase nuclei of AZT-treated cells. B. interphase nuclei of untreated HeLa cells. The bright spots, represent telomeric repeats.

α, β, and γ and high affinity for HIV-1 reverse transcriptase (9).

Our findings show that the nucleoside analog AZT is able to shorten the size of the telomeric repeats of tumor cells in culture during long term exposure. The shortening appeared to be linear with time in culture and was irreversible, because the length of the repeats is not restored after AZT exposure is discontinued. When the telomere length shortening takes place, some telomeric fragments remain intact, as shown by the smear associated with the blots in Fig. 1. After 15 passages the telomeres did not shorten sufficiently for a program of senescence to begin. Additionally, telomerase activity could not be detected in HeLa cells cultured with AZT for 13 passages and compared with untreated controls, with telomerase activity (B. Windle, personal communication). Our data is supported by previous publications showing that AZT, shortens the telomeres of *Tetrahymena* by decreasing the *de novo* telomere addition (10), decreases the telomere length in two immortalized human cell lines by telomerase inhibition (11), inhibits cell proliferation with senescent-like phenotype induction in cultured mouse fibroblasts (12) presents a growth inhibitory activity on cultured human breast cancer cells and rat mammary tumors (13) and shortens the telomeres of mouse exposed in utero (14).

The fact that telomere length was decreased in HeLa cells after long-term exposure to AZT without any evidence of senescence could be the result of different mechanisms. First and most importantly, the number of AZT treated passages could be insufficient for a senescence program to be triggered, a hypothesis that we are currently evaluating. Second, telomeres shortening to a critical length could induce a compensatory mechanism of preservation to prevent further losses. Third, an AZT-resistant phenotype could emerge as a result of selection by the treatment.

In conclusion, we demonstrated an irreversible telomere shortening by AZT treatment. We showed, for the first time that the shorter telomere length is maintained with no further telomere loss or addition, upon removal of AZT from the culture medium, suggesting the resumption of telomerase activity. AZT must be viewed as a telomerase inhibitor with potential anticancer properties.

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